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RESPIRATORY STUDIES OF SINGLE CELLS.[1] III. OXYGEN CONSUMPTION DURING CELL DIVISION

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The reference diver technique (Scholander, Claff and Sveinsson, 1952a) has made it possible to study in detail the oxygen consumption of large single cells. In a previous paper (Scholander, Claff and Sveinsson, 1952b) we reported observations on single protozoans, and in the present study we present data on the oxygen consumption during the first four cell divisions of the fertilized eggs of two sea urchins, *Strongylocentrotus purpuratus* and *S. franciscanus*, a sand dollar, *Dendraster excentricus*, and the echiuroid worm, *Urechis caupo*.

During mitosis a series of events takes place, all of which require energy in one form or another. These events could conceivably occur within the limits of a perfectly steady total flow of energy expenditure or they might present themselves as bumps or cycles on top of a "basal" energy exchange. The gross process of cleaving one lump of protoplasm into two or more pieces is one phase which could readily be expected to be associated with such an excess oxygen intake. In aerobic eggs the energy necessary for mitosis may in the last analysis be oxidative, and it is therefore natural to look for cycles in the oxygen consumption correlated to the anatomical events. Many authors have looked for such cycles in the oxygen consumption with both positive and negative results. For a complete survey the reader is referred to the excellent monographs by Needham (1931) and Brachet (1950).

Gray (1925) in two mass run measurements on *Echinus esculentus*, using a Barcroft respirometer, found no cycling in the oxygen consumption. By re-calculating and plotting his data according to the procedure which is described below, it is apparent that in one of his runs there are actually very slight accelerations in the rate associated with each of the three cleavages.

Runnström (1933a), using the Warburg apparatus, in mass runs with *Paracentrotus lividus*, found an increase in oxygen consumption starting at the appearance of the cleavage furrow. Brachet (1932, 1934, 1935, 1950), using in most experiments a Fenn respirometer, found cycling in the oxygen consumption of frog eggs (*Rana*

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fusca). In 34 runs with some 80 eggs per respirometer, he found that the peaks of his curves coincided with the appearances of the cleavage furrow.

Zeuthen (1949, 1950a, 1950b, 1950c) found cycling to be of constant occurrence in eggs of *Psammechinus miliaris, Strongylocentrotus franciscanus, Dendraster excentricus* and *Urechis caupo,* which were run several hundred at a time in modified Cartesian divers. The cleavage usually took place when the rate of oxygen consumption was at a minimum. In all of the echinoderms mentioned he found that the cycling increased markedly in intensity after the fifth to seventh division. Tang (1948) reports similar results on *Arbacia punctulata,* using the Warburg technique.

In all mass runs one must depend upon the degree of synchronism of the cleavages of a great number of eggs. There is necessarily some individual variability in the cleavage rhythm, and one faces the risk of promoting asynchronism by tension gradients developing in a sedimented cell mass. Whatever the reasons may be for differences in the individual rhythm of eggs, it would seem likely that the cell divisions would get progressively more out of step at each successive division. As amply demonstrated, especially by Zeuthen, the statistical approach has yielded extremely valuable information. A certain degree of blurring of the details is inevitable in mass determinations, however, and it is hence of interest whenever possible to supplement the information by runs on single eggs. This has hitherto been possible only for very large eggs, such as that of the frog. Zeuthen (1946) studied single frog eggs (*Rana platyrrhina*) in modified Cartesian divers, and found that cycling was a regular feature, with cleavage beginning at or slightly after a respiratory minimum. Cycling was also found to take place without cytoplasmic division.

In the present investigation we have undertaken to analyze on single eggs the oxygen consumption during cell division in several marine species. With the exception of *S. purpuratus,* Zeuthen (1949, 1950c) has studied the same forms in mass runs.

Material and Methods

The echinoderm material was secured at Friday Harbor, Washington, and *Urechis* at Pacific Grove, California.

Eggs and sperm from *S. purpuratus* and *Dendraster* were obtained either by spontaneous spawning or by injection of isotonic KCl solution. The spawning season was over for *S. franciscanus,* but enough eggs for our purpose were obtained by removing the ovary and shaking pieces of it in cold sea water, washing out a dozen eggs or more. The eggs were then rinsed free of all debris in several dishes of sterile and cool sea water. All dishes were kept cool.

Dendraster has a thick and colorful "furry" coating on the egg. This was stripped off from the fertilization membrane by passing the egg repeatedly through a narrow pipette opening.

The eggs and sperm of *Urechis* were taken by pipette from the genital pores. In this species all runs were performed on material from the same two animals, whereas in the echinoderms new pairs were used each time. Much difficulty was encountered with the *Urechis* eggs in the beginning because the egg adhered badly to the chamber wall and made the diver stick. It required many passings of the egg through a pipette to get rid of this stickiness.

At zero time the eggs were fertilized by stirring them with a fine glass tip which

had been touched to a cold and concentrated sperm suspension. The fertilization was followed under a dissecting microscope and as soon as the membrane had developed, a perfect-looking egg was picked for the run. This egg was transferred through two or three dishes of cold sterile sea water and loaded into the chamber, which was kept cold by having the supporting stopper chilled beforehand. The centrifuge was kept in the refrigerator. Sterile technique was used throughout (see Scholander *et al.*, 1952a).

Presentation of Data

In Figure 1 the cumulative oxygen consumption of the *Dendraster* runs has been plotted in $\mu\mu$l, omitting most readings that are less than three minutes apart. Under each of these curves we have drawn a straight line representing an average rate measured in $\mu\mu$l/min. We are particularly interested in seeing to what degree the observed curves deviate from the straight lines. To find this we calculated a series of figures for the average oxygen consumption corresponding in time to the observed

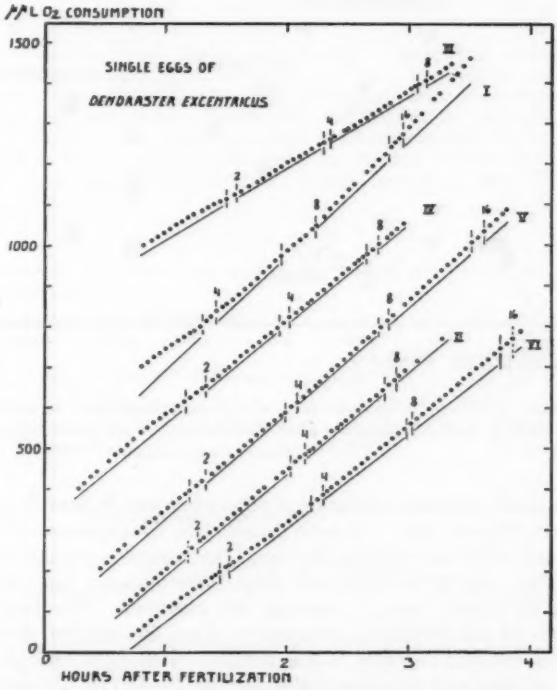

Figure 1. Cumulative total oxygen consumption during cell divisions in single eggs of the sand dollar, *Dendraster excentricus*. The beginning and end of each cell division are marked by vertical lines and the number of cells at the end of the division is indicated. Under each curve is drawn a straight line taken as the average rate of consumption. The position of each curve on the ordinate scale is arbitrary.

figures. The two sets of figures were subtracted from each other point by point, giving the absolute deviations from linearity in $\mu\mu$l. The deviations were plotted at their proper times and could now be sufficiently enlarged to permit a detailed analysis. The curve thus obtained is slightly skewed to the left because the values are plotted at an angle from the original ordinates. This effect is entirely negligible when the oscillations are as small as those presented. We have chosen this way of treating the data rather than recalculating the observations as a change of rate from point to point. The latter procedure has been most commonly used but is wasteful of good data if the observation points are very close together. The present curves represent simply the deviations from a straight line re-plotted on a magnified scale.

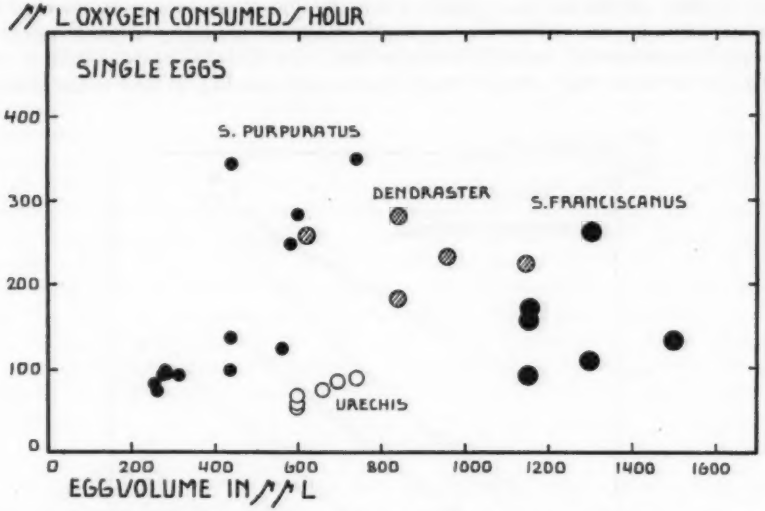

FIGURE 2. Cell volume in relation to rate of oxygen consumption in single marine eggs through the first three to four cell divisions after fertilization. Each point represents data from one egg. Tp. 15°–17°.

The results of the oxygen consumption determinations in four species of marine eggs are given in Figures 3–6. At each division the first appearance of a cleavage furrow is marked with one vertical line, and the complete separation of the cells with a second line. Up to the eight cell stage these lines are quite accurate, as all the cells could be clearly seen by turning the chamber. The beginning of the cleavage at the 8–16 cell division is accurate to about one minute, whereas the end, when the last cell finished dividing, is less certain. It is quite difficult to determine accurately the starting and finishing times for the 16–32 cell division, and for the next division (32–64 cells) they are even more difficult to see.

In Figures 3–6 the rate is represented by the slope of the curves. At the average rate they run horizontal. Above the second division a point has been located which corresponds to a 10% increase in the oxygen consumption in the period

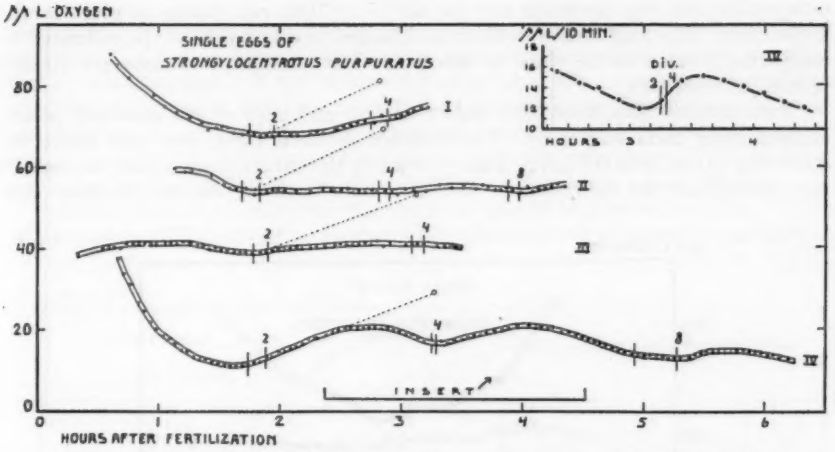

FIGURE 3. Deviations from a linear rate of oxygen consumption during cell division in single eggs of a sea urchin, *Strongylocentrotus purpuratus*. The average rate runs horizontal. A 10% increase of rate is indicated by the slope of the dotted lines. The ordinate units represent the absolute deviations given in μμl. The parallel lines indicate the spread of the observations and are drawn 1 μμl apart. The beginning and end of the cell cleavages are indicated by vertical lines and the digit at the end of the division gives the resulting number of cells. The position of each curve on the ordinate scale is arbitrary. Insert: Section of Curve IV recalculated as a rate curve.

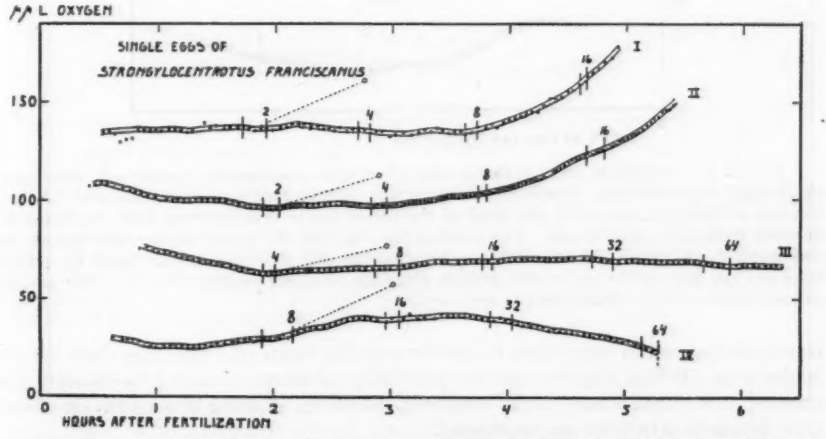

FIGURE 4. Deviations from a linear rate of oxygen consumption during cell division in single eggs of a sea urchin, *Strongylocentrotus franciscanus*. The average rate runs horizontal. A 10% increase of rate is indicated by the slope of the dotted lines. The ordinate units represent the absolute deviations given in μμl. The parallel lines indicate the spread of the observations and are drawn 2 μμl apart. The beginning and end of the cell cleavages are indicated by vertical lines and the digit at the end of the division gives the resulting number of cells. The position of each curve on the ordinate scale is arbitrary.

between the first two divisions, and the slope of a 10% rate change is given by the dotted line. The magnitudes of observed changes in rate can hence be estimated by comparing them with the slope of this line. The absolute deviations are directly plotted as ordinates.

Two parallel lines have been drawn one on each side of the observed points, encompassing their scattering. The distance between these lines and hence the scattering varies from 0.5 $\mu\mu$l to 2 $\mu\mu$l. Some of the curves show a slow increase of rate throughout the experiment. We dare not attach significance to these slow

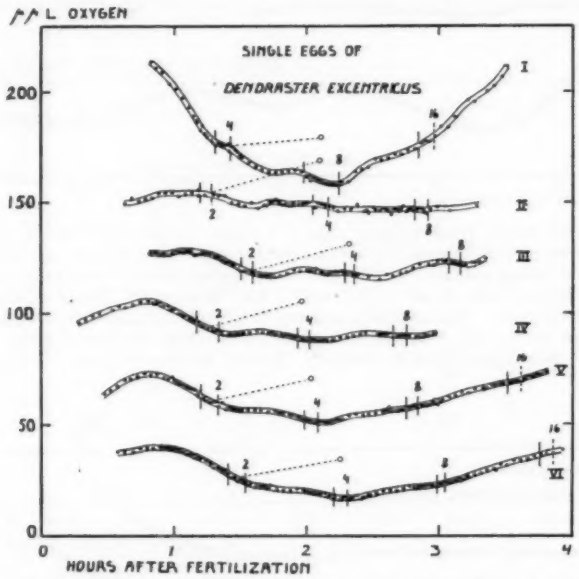

FIGURE 5. Deviations from a linear rate of oxygen consumption during cell division in single eggs of a sand dollar, *Dendraster excentricus*. The average rate runs horizontal. A 10% increase of rate is indicated by the slope of the dotted lines. The ordinate units represent the absolute deviations given in $\mu\mu$l. The parallel lines indicate the spread of the observations and are drawn 2 $\mu\mu$l apart. The beginning and end of the cell cleavages are indicated by vertical lines and the digit at the end of the division gives the resulting number of cells. The position of each curve on the ordinate scale is arbitrary.

trends as they are in magnitude inside the possible limits of a base line drift (Scholander *et al.*, 1952a, Fig. 7), and the possibility of infection cannot be entirely discounted. A slight steady drift does not, however, obscure the relatively short-time details in which we are interested.

RELATION OF EGG VOLUME TO OXYGEN CONSUMPTION

The egg cell diameter, not including the fertilization membrane, was measured, and from this the volume calculated, considering the cell a sphere. In *Urechis* the

measurement was taken after the caved-in pole had swelled out. In Figure 2 the egg volume is plotted against the average oxygen consumption during the first three to four cleavages. It will be seen that there is a very considerable variability in individual eggs as to both size and rate. In *S. purpuratus* the oxygen consumption varied two- to threefold for the same egg volume and there is on the whole poor or no correlation with size. *Paramecium* with the same range of size as these eggs had a metabolic rate about 5–10 times higher (Scholander *et al.*, 1952b).

Published data from mass runs for the most part fit well with our data. Tyler and Humason (1937) found an average O_2 consumption of *Dendraster excentricus*

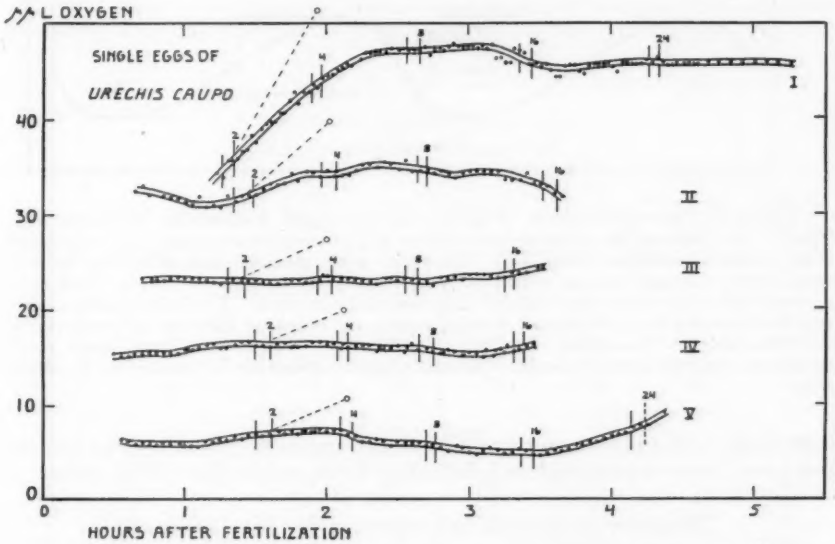

FIGURE 6. Deviations from a linear rate of oxygen consumption during cell division in single eggs of an echiuroid worm, *Urechis caupo*. The average rate runs horizontal. A 10% increase of rate is indicated by the slope of the dotted lines. The ordinate units represent the absolute deviations given in μμl. The parallel lines indicate the spread of the observations and are drawn 0.5 μμl apart. The beginning and end of the cell cleavages are indicated by vertical lines and the digit at the end of the division gives the resulting number of cells. The position of each curve on the ordinate scale is arbitrary.

of 208 μμl/hour at 15°. Lindahl and Holter (1940) gave an average egg volume of 584 μμl and an O_2 consumption (18°) of 155 μμl for *Paracentrotus lividus*. Borei (1948) obtained an average egg volume of 556 μμl for *Psammechinus miliaris*, with an O_2 consumption (18°) of 184 μμl. He found *Asterias glacialis* to have a very large egg of 2520 μμl with an O_2 consumption (18°) of only 250 μμl. *Arbacia punctulata*, with an egg volume of 190–260 μμl (Tang, 1931), has an oxygen consumption between 34 and 100 μμl (Krahl, 1950).

The oxygen consumption in our material of single fertilized eggs varies greatly

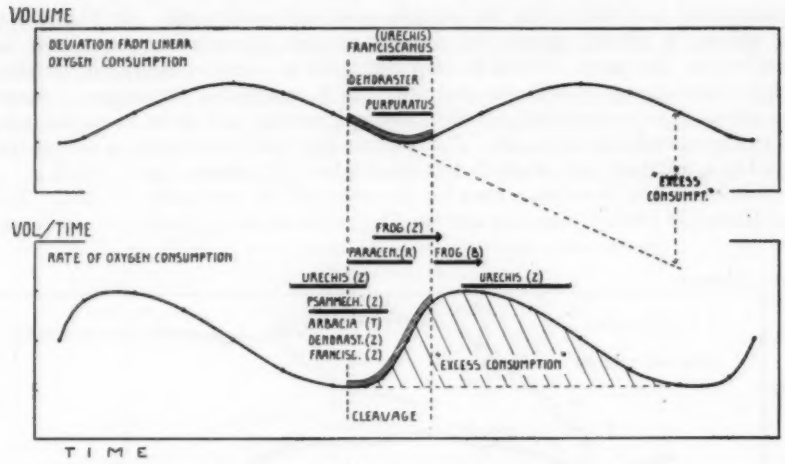

FIGURE 7. Generalized curves of cycling in the oxygen consumption of marine eggs. Upper: Curve showing the deviation from a linear rate of oxygen consumption such as obtained in the present investigation (Figs. 3–6). The region where cleavage occurred is given by horizontal lines. "Excess" oxygen consumption is indicated by a distance. Lower: This curve represents the upper curve recalculated as a conventional rate curve. The horizontal lines represent the regions where cytoplasmic cleavage started and ended, as found by different authors (B = Brachet, R = Runnström, T = Tang, Z = Zeuthen). The arrows (frog data) denote the region where the furrows started. "Excess" oxygen consumption is indicated by the shaded area.

individually with a poor or no correlation to size, and on the whole it seems doubtful that a near linear relation such as indicated by Smith and Kleiber (1950) exists.

RELATION OF OXYGEN CONSUMPTION TO CELL CLEAVAGE

The deviations from an average linear oxygen consumption have been plotted for fertilized eggs of four marine species (Figs. 3–6). Data pertaining to these runs and not given in the figures are presented in Table I.

Strongylocentrotus purpuratus. This species has very slowly cleaving eggs. Figure 3, curve IV shows a considerable cycling with total rate changes of about 20–25%. In this run the third cell division seemed delayed and showed poor synchronization, leaving one cell undivided. The cyclings decrease strongly in amplitude with time. If we interpret the increase of rate after, for example, the second division, as an "oxygen debt", it would, according to the construction given in Figure 7, amount to about 10–15% of the oxygen consumed during the period between the divisions. The rate increase by the cell division may well have other causes. In the insert on Figure 3 we have given the data before, during, and after the second cell division, recalculated from curve IV of Figure 3 as a conventional rate curve. The abrupt increase of rate at the cell division and the subsequent slow decline are clearly visible. We have not been able to find any clue from the data presented in Table I as to why this egg, IV, showed such a strong cycling.

Curve III shows an increase of rate at the first cell division of about 8%, suggestive of cycling. Curve II shows also an increase of rate at the cell divisions. Curve I exhibits a steadily increasing oxygen consumption and is inconclusive as to cycling.

S. franciscanus. In Figure 4, curves II, III, and IV, there are slight rate increases at the first and second divisions suggestive of cycling, but later there are none. When the single cell in curve III divided directly into four cells there was about a 15% increase in rate, or about twice that seen in II where the egg cleaved only into two. In curve IV the single cell sprang directly into eight cells with no more increase, however, than the cell which cleaved into two. It would seem likely that splitting into eight would represent more of a disturbance than splitting into two, and that it would show up in the oxygen consumption if the eggs were otherwise the same. This discordance may be explained by the fact that different eggs may

TABLE I

Oxygen consumption in marine eggs

Data pertaining to Figures 3–6

No.	Temp.	$\frac{\text{mm. Hg}}{\text{min.}}$	Factor $\frac{\mu l\ O_2}{\text{mm. Hg}}$	$\frac{\mu l\ O_2}{\text{hour}}$	Egg vol. μl	Water vol. μl	$\frac{\text{Water vol.}}{\text{Egg vol.}}$	$\frac{\text{Water vol.}}{\text{Bubble vol. of 200 } \mu l}$
			Strongylocentrotus purpuratus					
I	16°	0.9	1.8	98	280	40,200	144	201
II	16°	1.9	1.3	140	440	20,900	48	105
III	16°	2.7	0.6	98	280	11,800	42	59
IV	16°	1.6	0.9	83	255	18,700	74	94
			S. franciscanus					
I	16°	1.4	3.4	267	1300	84,600	65	423
II	16°	2.0	1.4	165	1150	33,500	29	168
III	16°	1.9	1.0	111	1300	22,400	17	112
IV	16°	1.9	1.5	171		36,200		181
			Dendraster					
I	15°	1.2	4.0	282	840	97,600	116	488
II	15°	1.2	3.4	252	600	81,500	136	408
III	15°	1.1	2.8	175		65,100		326
IV	15°	2.4	1.7	246	1150	37,800	33	189
V	15°	2.2	2.0	261		45,000		225
VI	15°	2.2	1.8	234	960	40,200	42	201
			Urechis					
I	17°	1.0	1.5	90	740	33,600	45	168
II	17°	1.0	1.3	78	660	29,200	44	146
III	17°	1.1	1.1	69	600	26,200	44	131
IV	17°	0.9	1.1	61	600	26,200	44	131
V	17°	1.1	0.8	55	600	17,800	30	89

cycle to a very different degree (*cf., S. purpuratus*). The later cell divisions in both runs, III and IV, were of normal appearance. At the end of these runs the cells were taken out of the chamber and placed in dishes, where they developed into perfectly normal-appearing plutei.

Dendraster excentricus. This is the only egg tested that seems to cycle regularly. The cycling is strongly damped and almost vanishes after the third division (see Figure 5, V and VI). The cleavage takes place after a low rate and is immediately followed by a rise. In curves I, V, and VI the cycling is superimposed upon a general increase in the oxygen consumption, which gives the impression of a persisting, stepwise increase at each division. It cannot be definitely excluded that this general increase may have been caused by baseline drift or possibly infection. Most cleavages were recorded with observations every minute, in order to see if any relatively rapid changes would take place immediately at the cleavage. No such changes were found.

Urechis caupo. This animal has a slowly metabolizing egg and great care was taken to minimize the spread of the observations, which in the later runs (Figure 6, III, IV, and V) were within 0.5 $\mu\mu$l. No cycling could be observed except in number II, where it appears rather clearly.

As a result of the above described runs on single eggs we may say that the cell division in many cases takes place without any visible change in rate of oxygen consumption. When a change does occur it is most commonly seen as a rather abrupt increase of rate at the first cell cleavage. Sometimes this increase subsides and is repeated at the next cleavage. We then get cycles in the oxygen consumption. These were clearly present only in *S. purpuratus* and *Dendraster*, and of inconstant occurrence in both. The cycles have a shape usually like the one drawn in Figure 7 (upper), rather than a regular sine shape. This generalized curve has been recalculated into a rate curve (Fig. 7, lower). It will be seen that this implies that the rise of rate is much more rapid than the decline (*cf.* insert of actual curve in Fig. 3). The cycling is in all cases strongly damped and usually vanishes after the second or third division. What happens after the fourth or fifth division we have not followed.

In the cases where we see a clear cycling in the O_2 consumption it seems that the cytoplasmic cleavage is closely associated with an increase in the rate of oxygen consumption (shaded line in Fig. 7). The elevated rate may persist for 30 minutes to one hour after the division is through and has been termed an "excess consumption." It may reflect an oxidative debt left over from the cleavage, although this took place under perfectly aerobic conditions. Direct calorimetry has so far not yielded sufficiently detailed information to clarify this point. Rogers and Cole (1925) found a 10% drop in the rate of heat production at the first cleavage of eggs of *Arbacia punctulata.* Trurnit (1939) found that eggs of *Psammechinus miliaris* cleaved during a temperature maximum. His technique has been questioned (Zeuthen, 1946).

The earlier data on mass runs where cycling has been found appear on the whole to agree with our findings, in that most authors have observed cleavage to start at or near a low oxygen consumption (Fig. 7). Zeuthen (1949, 1950c) states for all his marine eggs that the cleavage furrow appears when the respiration is decreasing. His drawings actually show a relation more like that presented in our

Figure 7. By comparing his Figures 1 and 3 (1950c), the cell division in *Urechis* evidently did not correlate well with the cycling, but occurred as we have plotted it in Figure 7, one position seemingly contradicting the other. He also found periodicity in a frog egg that did not divide, and hence considered that the extra oxygen consumption was not closely connected with the cytoplasmic cleavage. We have observed two *S. purpuratus* eggs that did not divide and these did not show any change in O_2 consumption at the time when they should have normally divided.

Influence of Low Oxygen Tension Upon the Respiratory Rate and Cleavage

Experiments were made to find out to what extent lowering of the oxygen tension during an experiment would influence the rate of oxygen consumption. For this purpose the water was saturated with air instead of oxygen, so that the egg would actually run itself out of oxygen. Figure 8 shows that the oxygen consumption keeps on at normal rate until the last one or two minutes, when it abruptly stops. If the egg uses 180 $\mu\mu$l/hour or 3 $\mu\mu$l/min., and uses up all of the oxygen, then the consumption would not start to drop until there are only 6 $\mu\mu$l left. Six

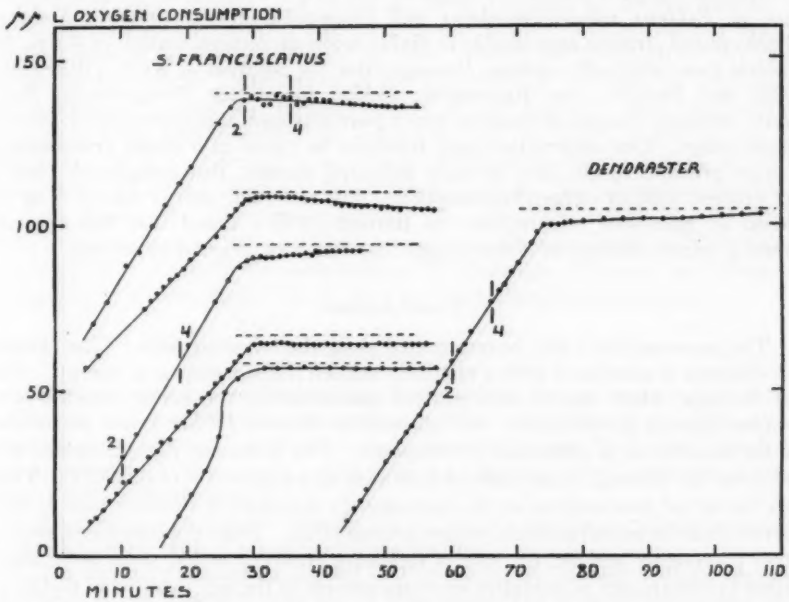

Figure 8. Oxygen consumption in single cells: experiments where the chamber water was charged with air instead of oxygen. At the sharp bend the cell has used up all of the oxygen. In the upper curve the oxygen ran out just as cell cleavage started. The cleavage continued without any oxygen consumption. The irregularities during this division are artifacts due to sticking of the pressure control, produced by the excitement of the operator. The position of each curve on the ordinate scale is arbitrary.

$\mu\mu$l oxygen will exert a pressure of 1/100 atmosphere when dissolved in 20,000 $\mu\mu$l water, and hence the oxygen consumption did not start to drop until a pressure of 7–8 mm. Hg was reached. This is in good agreement with Amberson's data on *Arbacia punctulata* (1928). In this species normal respiration keeps up until about 20 mm. O_2 tension is reached. The abrupt cessation of the oxygen consumption in our experiments is satisfactorily explained by the low O_2 capacity of the system compared to the relatively high rate of O_2 consumption.

Two of our curves showed a slight increase in flotation pressure after the cessation of oxygen consumption. This may be a sign of fixed acid formation with consequent release of CO_2. Perlzweig and Barron (1928) found lactic acid formation in *Arbacia punctulata* and Runnström (1933b) and Borei (1934) found fixed acid formation at fertilization in two other echinoderms. In two other curves we found, however, a slight decrease in the flotation pressure.

In one instance (Fig. 8, upper curve) the cleavage furrow appeared during the very minute when the egg ran out of oxygen. The division went on to full completion, however, in spite of a complete cessation of the oxygen consumption. Evidently, therefore, cell division in this species can be completed without oxygen consumption and in spite of a presumably very low oxygen tension. E. B. Harvey (1927) showed that complete absence of oxygen would stop the mitosis in *Arbacia*, and likewise in *Echinus microtuberculatus* and *Strongylocentrotus lividus*. Amberson (1928) found *Arbacia* eggs unable to divide below an oxygen tension of 4 mm. Hg. *Arbacia punctulata* will continue cleavage after the addition of KCN (Blumenthal, 1930) and Örström (see Runnström, 1933a) found that *Paracentrotus lividus* would continue cleavage if brought into a pure nitrogen atmosphere at or after the diaster stage. Our observation may therefore be taken as a direct demonstration of what previous results have strongly indicated, namely, that cytoplasmic cleavage can proceed without oxygen consumption. The anaerobic energy may well be furnished by adenosine triphosphate, as Barnett (1951) found that this substance would produce cleavage in *Arbacia* eggs that had been stopped by anoxia.

Conclusions

The presented facts may be interpreted along the following lines. The cytoplasmic cleavage is associated with a relatively sudden transformation of energy. Since the cleavage, when started, will proceed anaerobically, the energy needed is not supplied directly by oxidation. As indicated by Barnett (1951) it may be provided by the breakdown of adenosine triphosphate. The increased oxygen uptake at or following the cleavage would then be indicative of a restoration of the ATP. Therefore the actual time interval of the extra energy demands of cleavage may be much shorter than the period of extra oxygen consumption. Since the conditions are at all times thoroughly aerobic, the rate of oxidative recovery would seem to be limited rather by the amount of oxidative enzymes present in the egg than by an inability to obtain adequate supplies of oxygen. It is possible that cycling may depend upon a certain excess of enzyme, not present in a non-cycling egg. It may also be that the excess oxygen consumption reflects directly the energy requirements of the mitotic nuclear processes that take place throughout this period before the next cleavage. Some of these processes may well be associated with an increased energy

exchange and could hence produce cycling of the oxygen consumption even if the cytoplasmic cleavage failed to occur, such as observed by Zeuthen (1946).

In our experiments the cycling is always strongly damped. It almost vanishes after the two or three first divisions. Zeuthen (1949) has found that later on cycling increases very markedly. Even if it can be demonstrated that this late cycling also occurs in single eggs, there will still be a minimum of cycling after the second or third division, and hence there can be no simple correlation between the wave amplitude and the steadily increasing number of cell divisions. It seems reasonable to believe that in later divisions the synchronism gets progressively poorer, and the exact nature of the late cyclings, therefore, needs further clarification. We must emphasize, however, that cell division in individual cells very often takes place without any demonstrable cycling. Quite regularly, therefore, the energy requirements for the different phases are apparently fitted nicely together within the limits of a steady constant flow of oxidative processes. It seems necessary to await much more detailed information before we can form a consistent picture of the energetics of cell division.

We wish to express our appreciation to the officers of the Milton Fund for making the above work possible, and to Dr. A. Baird Hastings we extend our sincere thanks for his unfailing interest and inspiration. We are indebted to Dr. Eric G. Ball for advice on the interpretation of the data. Dr. T. G. Thompson at the Friday Harbor Oceanographic Laboratories of the University of Washington supplied us with every possible assistance during our stay, as well as many helpful suggestions. We acknowledge with gratitude the cooperation of Dr. E. F. Swan, Dr. A. W. Martin and Dr. R. L. Fernald, from whose experience in marine biology we benefitted greatly at Friday Harbor. Much credit is due to the efficient assistance of Mr. John Stevens in procuring equipment. We are much indebted to Dr. L. R. Blinks for providing us with excellent working facilities at Hopkins Marine Station, to Dr. A. C. Giese at the same station for invaluable assistance and advice on the handling of *Urechis*, and to Dr. C. B. Van Niel for inspiring discussion.

Summary

1. In the present investigation we have analyzed the oxygen consumption during the first two to five cell divisions in single eggs from three echinoderms (*Strongylocentrotus purpuratus, S. franciscanus, Dendraster excentricus*) and from the echiuroid worm *Urechis caupo*, employing the reference diver technique (Scholander, Claff and Sveinsson, 1952a).

2. There is poor correlation between the cell volume and the oxygen consumption in individual eggs, both of which vary considerably.

3. The oxygen consumption during cell division may proceed without measurable change in rate (usual in *Urechis, S. franciscanus*) or it may show cycling correlated with the cell divisions (*Dendraster* and *S. purpuratus*). If cycling occurs, the cytoplasmic cleavage is associated with an abrupt rise in the oxygen consumption.

4. The cycling, if present, is strongly damped and often disappears after two to three divisions.

5. When started, cleavage can proceed without oxygen consumption (*S. franciscanus*).

6. Our runs on single eggs reflect in some respects the results gained by mass runs, but we are able to supply a more exact picture with respect to timing, curve shape, and variations in individual cells.

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THE MECHANICS OF FLIGHT MOVEMENTS IN DIPTERA [1]

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Although the muscles that move the wings in flies and certain other insect groups are activated by the central nervous system, the individual wing movements are not. The wing rate is determined by the load on the flight muscles and was found to bear no relation to the action potentials in these muscles (Pringle, 1949; Roeder, 1951). Two theories have recently been proposed to explain the peripheral control of wing rate in flies. According to Pringle (1949) nerve impulses produce an alteration in the flight muscles so they may be excited on being stretched. By means of the thoracic skeletal connections between them, the shortening of one set of muscles stretches, and so excites, the antagonistic muscles. Boettiger and Furshpan (1950) considered the antagonistic muscles to be under constant tetanic stimulation during flight, the mechanical action at the articulation altering the muscle load so as to produce the oscillation. Both theories suggest that the mechanical action of the thorax controls the physiological response of the muscle.

In spite of the considerable morphological work on the thorax of the fly, relatively little attention has been given to the movements of the articulation induced by the contraction of the large indirect flight muscles. The studies of Lowne (1893) and of Ritter (1911) on the blowfly are the most complete. Mihalyi (1935/36), studying the house fly, has extended the interpretations of Lowne and Ritter to include a more accurate description of some of the mechanical features of the thorax and their relation to flight.

Although Lowne (1893) gives the most detailed description of the articulating structures, in several important aspects his interpretation of their action was in error, for his methods did not show how the parts changed position during flight. The study of living flies treated with CCl_4 has revealed these movements of the articulation and enabled us to obtain a better understanding of flight mechanics. No attempt is made here to produce a complete morphological study. Most of the structures discussed are illustrated in the papers referred to above. Our purpose is to determine how the mechanical features of wing movement are related to the operation of the neuromuscular mechanism.

The Action of CCl_4

In a short abstract (Boettiger and Furshpan, 1950) the effect of placing flies in CCl_4 fumes was described. There are first violent muscular spasms involving the whole body, with an apparent stiffening of the thorax. Usually anaesthetic flight, a well recognized phenomenon in certain insects, appears. With increasing

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action of the CCl_4, the flight tone (wing frequency) increases; then it becomes lower and irregular. Suddenly the wings stop in either the up or down position, usually the up position. That this abrupt stop is due to a mechanical inhibition to movement can be shown quite readily. If the fly is immediately removed from the CCl_4 and the wings moved toward the horizontal position, they will be pushed beyond the catch and will start to beat normally again. After longer exposures, normal movements will not start when the wings are depressed. Instead, when a critical point is reached, the wings will click without further effort into the opposite position and remain locked. In this movement the wings essentially follow the normal flight path with proper changes in angle of attack, indicating that the articulation is set by CCl_4 in the same manner as in normal flight. The most effective way to move the wings down from the up position is by pressure upon the scutellum. Pressure upon the posterior lateral region of the scutum will click the wings up. Small movements of the scutellum in a fly under CCl_4 will produce much larger movements of the wings than rather extensive squeezing of the thorax of an untreated fly.

Evidence was presented (Boettiger and Furshpan, 1951) that this click phenomenon is part of normal flight, and so the action of CCl_4 becomes a new tool for studying the mechanics of flight. The wings can be frozen in the up or down position and the relations of the parts studied. Furthermore one can click the wings up and down, thus revealing the dynamic aspects of the mechanism.

All observations reported here have been made on the large flesh fly, *Sarcophaga bullata* Parker. Other species of Diptera studied gave the same response to CCl_4, but the anatomical details of their articulations have not been investigated.

The Action of the Principal Parts of the Flight Mechanism

The scutellar lever and the action of the indirect muscles

The dramatic movement of the wings produced by pressure on the scutellum of a fly whose wing articulations are set with CCl_4 depends upon the action of the scutellar lever illustrated in Figure 1. This consists of the scutellum (a) and two lateral anteriorly projecting arms (b). These are strongly attached to the scutellum by the scutellar bridges (s) and the thickened ventral triangular pieces (t) hollowed out for articulation with the postnotum. The lever rests upon two earlike lateral projections of the postnotum which fit into special grooves (r) to make an articulation allowing rotation. Between these points the scutellum connects with the postnotum by a flexible membrane. The attachment of the lever dorsally to the main part of the notum is through the scutoscutellar suture and the thin flexible cuticle (l-h, Fig. 1) along the lateral arms extending to the clefts between the lever arms and the lateral scutum. The ventral border of each lateral arm is greatly thickened and bears the posterior notal process (not illustrated). The lateral arm extends to the axillary region on each side as a process (c). The x-shaped first axillary sclerite (e, Fig. 3C) sits upon this process. The outer ventral process of this sclerite fits into a special groove (u, Fig. 1C) in which it slides as the axillary sclerite twists on process (c).

The scutellum is so constructed that the movements of the lateral arms are identical. Damage to the thin cuticle forming the dorsal surface of the scutellum allows the two wings to be manipulated up and down somewhat independently. Flies so treated cannot sustain themselves in free flight.

The arms of this lever were called the great alar apophyses by Lowne (1893) and given equal importance with the anterior notal process (p, Figs. 1 and 2) in moving the wings. Ritter (1911) also described the action of the lever arm and termed it the postalaris, the anterior notal process being the prealaris. The terms

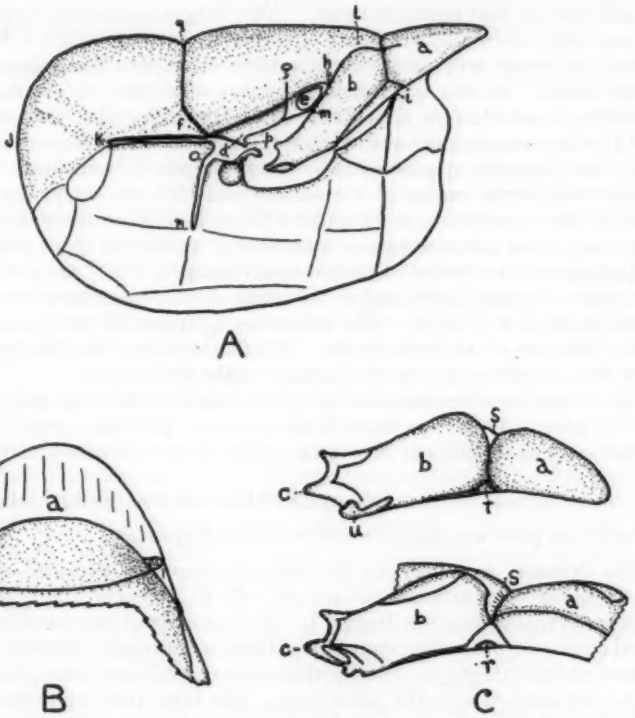

FIGURE 1. A. Left side of thorax of *Sarcophaga bullata*. B. Ventral view of scutellar lever dissected free from the thorax. C. Detail of lever arm and attachment to scutellum, outside above and inside view below. a, scutellum; b, lever arm; c, process articulating with the first axillary sclerite; d, anterior parascutum; e, posterior parascutum; f, junction of prescutal ridge, transverse ridge and parascutal hinge; g, end of parascutal hinge; h, point in line of attachment of notum with lever arm; i, point of rotation of the scutellar lever on the postnotum; j-k, fulcrum of the notum lever in the action of vertical muscles; l-m, line of attachment of notum with lever arm; n-o, lateral vertical cleft; p, anterior notal process; q, transverse ridge; r, articulating groove for attachment of postnotum; s, scutellar bridge; t, triangular structure supporting articulation of lever and postnotum; u, groove for a process of first axillary sclerite.

anterior and posterior tergal levers were used by Mihalyi (1935/36) who also described the rotation at the junction of notum and postnotum. These investigators all recognized the opposing action of the anterior notal process and the lever arm. In recent English literature the nomenclature of Snodgrass (1927) has been adopted. He does not describe the action of the lever arm. Pending some settlement of the

nomenclature by morphologists, the term scutellar lever is used here as it best describes the action of this part of the notum.

The scutellar lever by its simple rotary action and strong construction is designed to transmit the power of the indirect muscles to the wing root sclerites. In the downstroke the longitudinal muscle moves the hinge points (i, Figs. 1 and 2) forward, closing the lateral vertical clefts (n-o, Fig. 1A), while the upward movement of the notum opens the cleft (k-f). Mihalyi (1935/36) first explained the importance of these clefts in allowing proper movement of the thoracic parts. At the same time there is also a lateral expansion of the notum, especially at f (Figs. 1 and 2). The notum rides up and back as the hinge points (i) move forward; the scutellum tips down, and the lever arms move upward. If there is a definite fixed axis on which this rotation occurs, it must lie between points (i) and (l) for (i) moves forward and (l) moves back.

The part played by these structures in the upstroke is as follows. The vertical muscles reverse the rotation produced by the longitudinal muscle. In this action the whole notum anterior to the scutoscutellar suture serves as a lever with fulcrum at j-k (Fig. 1A) and moves the scutellar lever down by action at the attachment

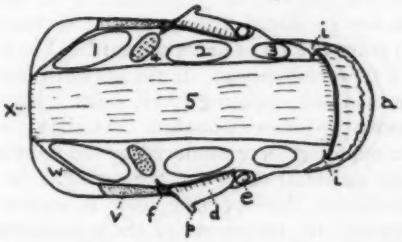

FIGURE 2. Horizontal section through the notum. Structures labeled as in Figure 1 with the following additions: v, prescutal ridge; w, chitinous supporting structure; x, anterior hardened plate; 1, first dorsoventral muscles; 2, second dorsoventral muscles; 3, oblique dorsal muscles; 4, tergal remotor muscles; 5, longitudinal muscles.

line (l-h-m, Fig. 1A). The first dorsoventral muscles shorten very little and move the lever at considerable mechanical disadvantage. They are effective, however, in producing lateral expansion at point f and stiffening the thorax. The second dorsoventral muscles act upon the lever with greater mechanical advantage and may also produce lateral expansion. The oblique muscles attach to the notum just medial to the lever arm at point 1 (Fig. 1A), and, although they are small muscles, must be quite effective in rotating the lever arms down. The vertical muscles, especially the oblique muscles, are arranged to pull posteriorly as well as downward and so tend to force the hinge points (i) back, opening again the vertical clefts (n-o) without at the same time straining the fulcrum region (j-k).

The generally accepted theory of the production of the up and down movements of the wing suggests that the longitudinal muscles expand the notum laterally on the downstroke, and the vertical muscles draw the notum inward on the upstroke. The recognition of the part played by the scutellar lever eliminates the necessity of assuming this action of the vertical muscles. A study of the insertion of these

muscles on the notum makes it unlikely that they could move the lateral notum inward. In fact Lowne (1893) suggested that the longitudinal and vertical muscles are only partly antagonistic in action, as both tend to produce lateral expansion of the thorax. There must be a continuous outward force produced by the notum and transmitted to the articulation through the anterior notal process. The significance of this force in flight mechanics is discussed in the next section.

The spring mechanism of the anterior notum

The strengthening of certain regions of the thorax was well illustrated by Mihalyi (1935/36), although he does not comment on its significance in flight. Referring to Figures 1 and 2, one can see these structural re-enforcements. Point f is joined to a similar point on the opposite side of the thorax by means of the strong transverse ridge extending over the notum. From f two parallel ridges (prescutal) connected by a stiff membrane (v, Fig. 2) arise and extend anteriorly. A supporting structure (w) connects them with the anterior boundary of the thorax where it joins a hardened plate (x). The transverse and prescutal ridges strengthen the anterior notum so it will resist any force tending to move point f inward.

The attachments of the longitudinal muscle and of the anterior vertical muscles to the anterior notum are so placed that as these muscles shorten, the thorax is put under tension and point f tends to move outward. The thorax and the muscles act together to form a powerful spring. If the outward lateral force produced by this spring is greater than any opposing force, point f, the parascutum (d), and the anterior notal process (p) move outward. As shown below, during the first part of each stroke, an opposing force produced by the scutellar lever moves point f inward, thereby storing potential energy in the spring. At a critical point in the movement of the articulation, this opposing force is suddenly removed. The recoil of the spring completes the movement of the articulation and the wing. The snap action of the scutellum in wingless flies executing flight movements, as previously described by Boettiger and Furshpan (1951), demonstrates the operation of the spring mechanism. The click phenomenon seen in living flies under CCl_4 also results from its action.

The movements of the scutellar lever and of the lateral notum are transferred to the articulating portions of the notum and to the axillary sclerites of the wing base, and thus produce the characteristic pattern of wing movement. The wing cycle then results from the interaction of the anterior parascutum and the anterior notal process, the first and second axillary sclerites, and the mesopleural wing process. Their functions will now be described.

The anterior parascutum and the anterior notal process

The anterior notal process arises, as shown in Figures 1 and 2, from the thickened outer border of a shelf-like structure, formed of the anterior parascutum (d) and the posterior parascutum (e) (terminology of Lowne). Dissecting from the inside, one can see that the anterior parascutum articulates with the main part of the scutum by a concealed hinge (f-g), which allows the shelf to be moved easily up and down. Posteriorly the shelf is connected with the lever arm at h-m. The cleft between the lever and the parascutal shelf, called the tergal cleft by Mihalyi, allows

somewhat independent movement of these two structures, a fact which is important in the production of the basic movement cycle.

As noted above, contraction of either the longitudinal or the vertical muscles will produce lateral expansion of the thorax at point f. This force is transmitted to the articulation by the anterior notal process. In Figure 3 (A and B) the connection between the main part of the notum and the articulation is shown. The existence of the flexible hinge connection (h), not previously described, makes the presently accepted theory of the cause of the down movement of the wings highly

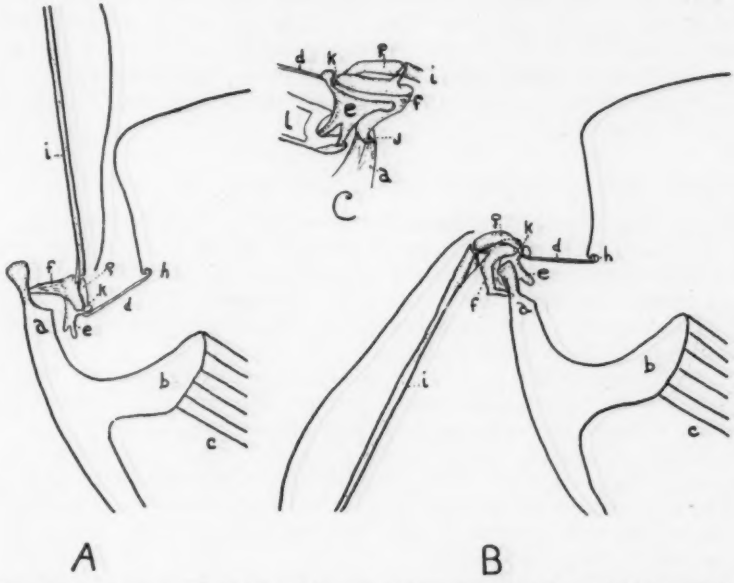

FIGURE 3. Cross sectional view of thorax showing details of the articulation of the right wing. A. Wing in up position, anterior view. B. Wing in the down position, anterior view. C. Posterior view of the axillary sclerites of right wing showing their relation to the mesopleural process, the lever arm and the anterior parascutum. a, mesopleural process; b, pleural apophysis; c, anterior pleurosternal muscle; d, anterior parascutum; e, first axillary sclerite; f, second axillary sclerite; g, base of radial vein; h, hinge; i, radial vein; j, hook articulation; k, point of articulation of anterior notal process, first axillary sclerite, base of radial vein and second axillary sclerite; l, end of the lever arm.

unlikely. Lateral expansion of the notum cannot possibly move the articulation from position shown in Figure 3A to that in Figure 3B as this theory predicts. Expansion of the notum, laterally, would move the hinge outward. However, due to rotation occurring at the hinge, the anterior notal process would not be moved out or up and the wing would remain in the up position (see Fig. 3). Through the anterior notal process the lateral force is focused on the wing articulation. The change in direction of this force necessary to wing movement is produced not by alternate flattening and arching of the notum, but by the scutellar lever. The lever

acts upon the anterior notal process partly through the connection between the lever arm and the parascutal shelf (m-h, Fig. 1) and partly through the first axillary sclerite (e, Fig. 3C).

The first axillary sclerite

The transformation of the movements of the lever and the anterior notal process into the complex wing cycle depends upon the action of the first and second axillary sclerites. Figure 3 (B and C) shows the relationships of the sclerites when the wings are down. The lever exerts its main effect upon the articulation through the first axillary sclerite. The large inner dorsal process of this sclerite hooks into the anterior notal process at k so that as the lever moves up and down, both k and the parascutal shelf are also moved. The second axillary lies between the two dorsal arms of the first axillary. The movement of the second axillary is largely determined by the first axillary.

The second axillary sclerite and the mesopleural wing process

Lowne (1893) described the relation between the second axillary and the mesopleural process in the blowfly. He stated that it is difficult to assign any function to this relation. Ritter (1911) believed the second axillary strengthened the joint by tying together the upper and lower parts. Snodgrass (1927) noted that the second axillary sclerite usually articulates at the side of the mesopleural process, but did not discuss the implications of this fact.

The second axillary is hooked to the side of the mesopleural process (Fig. 3C) and does not disarticulate as suggested by Lowne. The connection is a very strong one and acts almost as a universal joint to allow the complex wing cycle. The second axillary, through its relation to the radial vein, plays an important role in directing the wing. As shown in Figure 3, the base of the radial vein (g) articulates at k along with the anterior notal process and the first axillary. It also makes secondary membranous connections with the second axillary so that rotation of this sclerite moves the wings up and down.

The relation of the base of the radial vein to the second axillary is controlled in part by the direct muscles that fold the wing back over the body and bring it forward into flight position. As the wing folds back the base rotates forward. This arrangement allows some folding of the wing during flight without essentially disturbing the second axillary, the position of which plays an important role in the basic movement of the wing.

As noted above the outward force produced by the notum is transmitted to point k by the anterior notal process. This is opposed by the contraction of the anterior pleurosternal muscle (c, Fig. 3), which is one of the direct flight muscles. As this muscle draws the mesopleural process inward, a force is transmitted to articulating point k by the second axillary sclerite. The inward movement of the mesopleural process is of prime importance in setting the articulation for flight. It occurs in CCl_4-treated flies and is necessary for the production of the click phenomenon.

The position of the mesopleural process determines the range of movement of the lever arm. This is why the movements of the wings of CCl_4-treated flies are more extensive than those of untreated flies. The two lateral arms of the scutellar

lever must move the same for they are strongly connected through the scutellum. Therefore it is important that the muscles exert equal forces on the two mesopleural processes. A provision for this appears to be present. The pleurosternal muscles have a common origin on the flexible sternal apophysis and they tend to act as a single muscle, the ends of which are attached to the two mesopleural processes.

The mesopleural process with its articulating second axillary sclerite opposes the lateral notum with its hinge-attached parascutum. When these structures are properly set by the action of the indirect muscles and the appropriate direct muscles, the basic movement cycle can be produced by the action of the scutellar lever. It is the action of these structures that controls the loading of the fibrillar muscles and makes possible the fast flight movements.

The Production of the Basic Flight Movements

Two conditions are required for the production of the basic flight movements: (1) the activation of the spring mechanism by tension in the indirect muscles, and (2) the inward movement of the mesopleural processes. At the beginning of flight, forces are developed to move the hook articulation of the second axillary inward, and the hinge of the parascutum, which is attached to point f, outward. In a resting fly, point k, Figure 3, is above a line connecting the hinge and the hook articulation. Consequently, as these structures approach one another, point k will move up and the wings down. Records of movements of the scutellum show that in a typical start, a small down movement, which is relatively slow, occurs first. Then the wings are suddenly and rapidly brought up. Subsequent movements occur at a frequency normal for the wing load. The flight mechanism requires a starter, and it is probable that the initial fast up movement is caused by the tergal remotor (4, Fig. 2). This muscle is a vertical muscle but is not fibrillar in structure and responds as normal tubular muscles do.

The positions of the articulating elements when the wings are in the up position are shown in Figure 3A. As the longitudinal muscle shortens, the scutellar lever moves the first axillary sclerite (e) upward. This acts on point k, which can move up only if the mesopleural process and the parascutal hinge are forced apart. By this means, potential energy is stored in the spring mechanism, to be released when point k rises above a line connecting the hook articulation and the hinge. This occurs at the critical point. The recoil of the spring drives the wings down and the articulation into the position shown in Figure 3B. On the up movement of the wings the scutellar lever brings point k down. Again energy is stored in the spring mechanism and released at a critical point.

The critical point on the downstroke occurs when point k rises above the line of force between the hook process and the hinge and on the upstroke when point k moves below this line. Referring to the discussion of the action of the indirect muscles and the movement of the notum, it can be seen that the parascutal hinge must move up on the downstroke and down on the upstroke. This movement alters the position of the line of force between the hook and the hinge. The critical point therefore is at different wing positions on the up and down strokes. The elastic recoil force takes over at least part of the muscle load before the midpoint (resting length of the muscle) is reached.

At the critical point during the movement, the recoil force is maximum, but the major component of the force is not directed to produce rotation of the second axillary. As the movement continues, however, a greater and greater component of the recoil force is in the direction of rotation, while at the same time the force is becoming progressively less as the elastic energy is used up. Vertebrates in their skeletal movements make use of this same mechanical principle (Elftman, 1941).

In flies under CCl_4 an excessive force is produced by the direct muscles (and perhaps the indirect muscles). The indirect muscles are unable to overcome the force and the wings stop in one of the two stable positions, up or down. A similar condition results from high frequency stimulation of the ganglion (Boettiger and Furshpan, 1951). The fast stops in tethered flight reported by Boettiger and Furshpan (1950) and Roeder (1951) can be explained in the same way.

The Wing Cycle

To sustain the fly in free flight, the basic movements just described must initiate a cycle of wing movements aerodynamically designed to generate a propelling force, the magnitude and direction of which are adjustable by the insect. In this action, the indirect muscles, the direct muscles, and the structural features of the thorax are all concerned. Several authors have concluded, from the histological difference between the direct and indirect muscles, that the direct muscles cannot produce the rapid phasic changes of a single cycle. The observation that the wings of CCl_4-treated flies can be moved through a cycle by simply moving the scutellum is additional evidence for this idea, as no adjustment of direct muscle tension is possible during this movement. Mihalyi (1935/36) suggested that the indirect muscles, through the special arrangement of the three main articulating structures, can produce the complex wing cycle of straight level flight. He lists these structures as the mesopleural process, the anterior notal process and the lever arm process. The relative movements of these structures determine the movements of the axillary sclerites. These movements are able to produce a normal cycle, however, only when the articulation is set by the action of both the direct and indirect muscles.

The base of the radial vein of the wing is locked into the joint at k, Figure 3A, along with the first axillary and the anterior notal process. The important second axillary, although not articulated at k, is closely tied to this point and to the first axillary. The movements of point k govern the wing cycle. Through secondary membranous connections with the plate-like part of the second axillary, the wing moves with the rotation of this sclerite produced by the movement of k. Point k can move in the three directions, up and down with the lever, forward and back with the anterior notal process, and in and out with the rotation of the parascutum and the movement of the lateral notum.

Referring to Figure 3, one can analyze to some extent the movements that produce the wing cycle. When the wing is in the down position, it is held at the extreme forward point of the cycle in a vertical plane along side of the thorax. The articulation in this condition is shown in Figure 3 (B and C). As the vertical indirect muscles shorten, the scutellar lever process (1, Fig. 3C) starts to move down. The parascutum (d) does not move much at first, as it is held by the tension between the mesopleural process (a) and the parascutal hinge (h). Therefore, the second axillary first tips back, rotating the base of the wing so as to raise the an-

terior margin and depress the membranous part. As the lever continues to move down, point k moves more rapidly. The wing flips up and back in an effective stroke with the anterior margin of the wing leading. Before coming into the up position, a rotation of the wing base depresses somewhat the anterior wing margin. This is shown by the fact that in CCl_4-treated flies the wing locks into the up position with the anterior margin of the wing leading in a position to begin the downstroke. This rotation during the upstroke is due to the forward movement of point k, produced by the anterior notal process, and the twisting of the second axillary, resulting from the inward and downward movement of the lever arm.

The downward movement of the wing is also initiated by the scutellar lever as it is moved up by the shortening of the longitudinal muscles. The first axillary is lifted in such a way that the second axillary twists to tip the anterior margin of the wing down. Then point k flips up and the wing, held at the proper angle of attack, moves down. During the down stroke, the backward movement of point k and the forward movement of the lever arm process twist the second axillary, thereby insuring the full forward sweep of the wings. In CCl_4-treated flies the wing locks in the down position with the anterior margin closer to the body than the membranous portion as shown in Figure 3B. The wing is set as at the end of a downstroke, instead of ready for the upstroke. At the beginning of the upstroke the anterior margin of the wing must be raised while the membranous portion is depressed, as noted above. The upstroke is more complicated. The articulation is set to raise the anterior margin of the wing at the beginning of the upstroke and depress it at the end. Records of movements of the scutellum during a quick stop reveal a basic asymmetry in the articulation. In a quick stop on the downstroke there is some movement of the scutellum before the stop point is reached. On the upstroke the stop occurs almost at the extreme down position of the scutellum (Boettiger and Furshpan, unpublished).

The base of the wing makes secondary connections to the driving mechanism. The most important one is with the posterior notal process, which arises from the ventral border of the scutellar lever arm. This undoubtedly aids in tipping the wing and supporting the base against the effects of air pressure. However, cutting the posterior notal processes on both sides does not destroy the fly's ability to maintain itself in free flight.

The Mechanical Limits to Movement

Normally, more energy is released than is necessary to complete the movement (Boettiger and Furshpan, 1951). The freely moving lever is stopped by definite mechanical limits, which appear, however, to have some elasticity. The kinetic energy of the moving wings may be partially recovered by this means, as suggested by Chadwick (1951). The lever arm can move down without hindrance until its ventral ridge contacts the pleural sclerites. This first occurs quite near to point i (Fig. 1), as the arm is set at an angle so that it moves inward as it moves down. The second axillary sclerite seems to limit the up movement of the lever. As the lever pushes against the first axillary, the second axillary moves into the position shown in Figure 3C. The hook articulation with the mesopleural process prevents any further upward movement of the second axillary and hence of the first axillary and the lever. The presence of mechanical stops may increase the effi-

ciency of the mechanism by eliminating the necessity for doing negative work in stopping each movement (Fenn, 1930).

The Steering Mechanism and the Control of Amplitude

The movements of the lever arms and therefore of the articulation must have the same amplitude on the two sides. The construction of the scutellum does not allow one arm to move more than the other. If the wings are not drawn forward into flight position when a fly is under CCl_4, the amplitude of the wing movement when the scutellum is depressed is quite small. Even though the wings merely tip down, the click action is present and the lever arms move the full limit. The first axillary does not twist outward and forward as it does on the downstroke when the wings go through their full amplitude. As the wings are brought forward by direct muscles, the amplitude increases. Frequently, mounted flies will show erratic movements and hold one wing back while the other moves with normal amplitude. They spin around toward the side of the smaller movement. In a similar manner, a free flying insect can alter its direction without changing the action of the indirect muscles or of the scutellar lever. Should the fly wish to rise or fall, the tension in the proper direct muscles is adjusted, altering the wing cycle equally on both sides and again requiring no change in the driving mechanism.

Chadwick and Williams (1949) and Chadwick (1951) have made a quantitative study of wing movements in Drosophila at various air densities. Reducing the density of the air lowers the wing load and increases both frequency and amplitude. The increase in frequency is proportional to the increase in amplitude. This proportionality may be interpreted to mean that the increased amplitude observed at low air density is the result of the greater momentum of the faster moving wings. With greater momentum the articulation moves further into the elastic mechanical stop. In addition, each wing will also bend more when its articulation is suddenly stopped and this would be recorded as an increase in amplitude. Changes in frequency and amplitude may, therefore, be produced experimentally without alteration in the action of the basic neuromuscular mechanism. Roeder (1951) has found that reducing muscle load by removal of the wings results in a decrease in frequency of thoracic potentials. This suggests that receptors, responsive to wing load, can exert some control over the indirect flight muscles.

The amplitude of wing movement must be controlled to some degree by the central nervous system. In the first place, the wings may be held forward or back as described above so that their amplitude is large or small. Secondly, the tension between the mesopleural process and the parascutal hinge which determines the spring action of the notum can control wing amplitude. If this tension is large, the indirect muscles must, in overcoming this force, store a great amount of energy in the spring mechanism. The recoil action will then throw the articulation to the limit and so give maximum wing movement. On the other hand, if the tension is small, less energy is stored and the recoil may not move the articulation to its limit. In a normal stop there is a progressive decrease in amplitude of wing and articulation. This is apparently due to the relaxation of the direct muscles controlling the position of the mesopleural processes. Records show that during continuous flight under constant conditions, the amplitude of movements of the scutellar lever is remarkably uniform. Occasional changs in amplitude may be accounted for by al-

tered tension between the mesopleural process and the parascutal hinge. The indirect muscles by controlling the spring action of the notum may contribute to these amplitude changes.

Summary

1. The mechanics of insect flight are usually studied by squeezing the thorax so as to imitate the action of the indirect flight muscles. In flies certain elements of the articulation are not properly set in such experiments, so the interpretations made are not accurate. That CCl_4 sets the articulations of flies as in normal flight is shown by the dramatic wing movements easily produced in these insects. This report is based on the study of CCl_4-treated flies and outlines the main features of the mechanics of wing movement. Much additional physiological evidence for the interpretations given here has been accumulated and will be reported separately. The abstracts referred to state some of the experimental results.

2. The secret of the peripheral control of wing rate certainly lies in the indirect flight muscles. These muscles are the power plants of the wings, operating in essentially an all-or-none fashion. It is shown here that they play little part in steering or in amplitude changes. They operate between definite mechanical limits and so do not halt each other's movement as in a conventional antagonistic system.

3. The anterior notum is shown to have a spring action which unloads the muscle at a critical point and so plays a basic physiological role. If the recoil of the notum alone is capable of completing the movement, the indirect flight muscles would not be required to exert any tension at their resting or shorter lengths. The energy would be put into the mechanism almost isometrically and it would not be necessary to assume high tension in a rapidly shortening muscle.

4. By a combination of the mechanical properties of the thorax and the physiological properties of the fibrillar muscle the peripheral control of wing rate is achieved.

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HORMONAL REGULATION OF THE DISTAL RETINAL PIGMENT OF PALAEMONETES

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There have been several investigations (Parker, 1897; Welsh, 1930; Bennitt, 1932; Kleinholz, 1936) of the control of the migration of the distal retinal pigment of the prawn, *Palaemonetes*. Bennitt demonstrated that when one eye of *Palaemonetes* was covered and the other eye illuminated, both eyes assumed to a greater or lesser extent the light-adapted state. This suggested a hormonal control of the retinal pigments. Later, Kleinholz (1936) induced the distal retinal pigment of *Palaemonetes* to assume the light-adapted position by injecting eye-stalk extract into dark-adapted prawns, thus suggesting the existence of a light-adapting hormone whose source was the eye-stalk. That this was the normal controlling mechanism was indicated by the fact that eye-stalks of light-adapted animals were more bountifully supplied with this factor than those of dark-adapted ones.

The results of the experiments of Brown, Fingerman and Hines (1952) and Brown, Webb and Sandeen (1952) in which dark-adapted prawns are exposed to 250 ft.-c. light flashes both with and without earlier conditioning stimuli have not only given strong support to the hypothesis that there is a light-adapting hormone normally active in regulating the position of the distal retinal pigment of *Palaemonetes*, but have also indicated the action of a dark-adapting hormone. Prawns kept in darkness overnight exhibit quite a different rate of re-dark-adaptation when returned to darkness following a one-minute, 250 ft.-c. light exposure early in the morning than do prawns which earlier the same day had received the normal stimulus of dawn illumination before being placed in darkness and later submitted to the one-minute light exposure. Such a difference was postulated to be due to different degrees of availability of a dark-adapting factor.

The present experiments were undertaken: (1) to establish more definitely the presence of a dark-adapting hormone, (2) to determine the sources of the light- and dark-adapting hormones within *Palaemonetes*, and (3) to elucidate further the normal roles of these hormones in light- and dark-adaptation.

Materials and Methods

For the following experiments specimens of the common prawn, *Palaemonetes vulgaris*, were usually collected daily from the Eel Pond in Woods Hole, Massachusetts. In the laboratory the stock supply of animals was kept in aquaria in running sea-water from which the animals for each experiment were randomly taken without regard to size or sex. The experiments were conducted during the months of June and July.

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The influence of the various experimental procedures on the distal retinal pigment in the eyes of these prawns was observed through the use of a method of direct measurement of the position of this pigment in the intact, living animal (Sandeen and Brown, 1952). The method consisted essentially of holding a prawn on the stage of a dissecting microscope and viewing the eye under a relatively high magnification using transmitted light. With an ocular micrometer the width of the transparent area, which is a direct function of the degree of light-adaptation, can be measured from the cornea to the distal margin of the pigment. Since the animals varied considerably in size, the degree of light-adaptation was always expressed as a ratio of this width to the total distance from corneal surface to the proximal edge of the retina using arbitrarily as a marker the black spot, or ocellus, which is apparent on the dorsal aspect of the eye-stalk. This ratio will be referred to as the distal pigment index.

In all experiments in which *Palaemonetes* were placed in the dark room or exposed to various light intensities, white enamelled pans with a bottom diameter of approximately 7 inches were used. Sea water was placed in the containers to a depth of about 1½ inches. To prevent overcrowding no more than 12 to 14 animals were ever placed in a single pan. The various illuminations which were used were obtained with incandescent lamps placed at adjusted distances directly above the animals. The resultant illuminations were measured with a Weston photometer.

In the experiments in which prawns were injected with extracts of various organs the extracts were prepared in the following manner. The organs were removed with the aid of a dissecting microscope, from normal animals taken from the stock supply, and transferred to a small container of sea water. When a sufficient number of the organs had been obtained they were placed with a minimum of water in a glass mortar with a finely ground surface and while still moist were triturated as completely as possible with a glass pestle. Sea water was then added to make up the desired concentration, mixed thoroughly, and the extract drawn into a one ml. hypodermic syringe graduated in hundredths. The whole procedure was carried out very rapidly and the extracts used immediately. In no case did more than 30 minutes elapse between the beginning of the dissections and the injections.

Comparison of the Responses of One-Eyed and Normal Animals

In the first series of experiments the influence of the removal of one eye on the distal retinal pigment of the remaining eye was determined. In each of six experiments five *Palaemonetes* were taken at random from the stock supply and placed in a small amount of sea water in a white enamelled pan at an illumination of 25 to 50 foot-candles. One eye-stalk was carefully removed with a pointed scalpel under a dissecting microscope and the eye-stub cauterized immediately. The position of the distal pigment of each eye to be observed in the experiment was determined prior to the operation and then at 5, 10, 15, 30, 60, 90, 120 and 240 minutes following the operation. The first three of these experiments were conducted in the morning and the last three in the early afternoon. No significant difference in responses was found for these two times of day.

The average distal pigment index of the five animals in each experiment was calculated for each time that a determination was made. These averages were used to calculate a final distal retinal pigment index for each time that a reading was

made for all six experiments. Therefore, each final index at any given time represents an average of the condition for 30 animals. The final average indices of the six experiments were used to prepare Figure 1(A). It can be seen that the distal pigment index drops from 0.18 to 0.16 in 30 minutes and remains at this lower level for the duration of the experiment, a period of four hours. It had previously been determined (Sandeen and Brown, 1952) that at this illumination the distal pigment index of normal animals has a value ranging between 0.18 and 0.20. This is represented in Figure 1(A) by the broken line above the curve just described. Thus, under conditions of equal illumination the distal retinal pigment of *Palaemonetes* with only one eye-stalk is maintained at a lesser degree of light-adaptation than that in normal animals with their two eye-stalks.

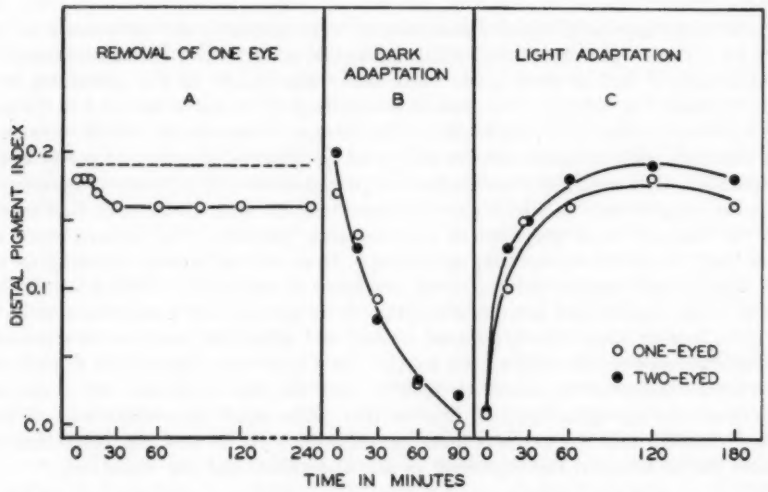

FIGURE 1. A. Change in the degree of light adaptation of the distal retinal pigment following removal of contralateral eyestalk. B. Comparison of rates of dark-adaptation in one-stalked and normal prawns. C. Comparison of rates and degrees of light-adaptation in one-stalked and normal prawns.

In a second series of experiments the rate of dark-adaptation of one-eyed prawns was compared with that of normal prawns. Approximately twenty-four hours before each experiment specimens of *Palaemonetes* were taken from the stock supply of animals and one eye of each was removed and the eye-stub cauterized. These animals were then returned to an aquarium with running sea water until the experiment was conducted the following day.

In each of the two experiments which were performed, 10 one-eyed *Palaemonetes* were placed in each of five pans. Similarly, normal animals were taken at random from the stock supply and 10 were placed in each of five pans. The distal pigment indices of 10 one-eyed animals and 10 normal animals in one of the pans of each group were determined. These two pans together with the others in each group

were then placed in the dark room. In the first experiment distal pigment indices of one-eyed and normal *Palaemonetes* were determined at 15, 30, 60 and 90 minutes after being put into the dark. In the second experiment distal pigment indices were determined only at 30, 60, and 90 minutes. At each of these times one container from each group was removed from the dark room and the distal pigment indices of the animals were determined. Following the determination these animals were discarded; a different container of animals was used for each successive determination.

The average distal pigment index for 10 animals of each group at each time that a determination was made was calculated. Average values were then obtained for both experiments and these were used to prepare Figure 1(B). Since a distal pigment index determination was not made at 15 minutes in the second experiment, the average index shown in the graph is that obtained in the first experiment.

It can be seen from Figure 1(B) that there is no significant difference between the rates of dark-adaptation of these two groups of animals. The presence of one eye-stalk is sufficient to permit an animal to dark-adapt at the normal rapid rate.

In a third series of experiments the rate of light adaptation of one-eyed animals was compared with that of normal animals. In each experiment a group of six pans containing 10 normal animals each and a group of six similar pans with 10 one-eyed animals in each were left in darkness overnight and then brought abruptly into an illumination of 250 ft.-c. where the course of light-adaptation of the animals was followed.

The distal pigment indices of 10 normal animals and 10 one-eyed animals were determined at the time the two groups were brought into the light and at 15, 30, 60, 120 and 180 minutes thereafter. The average value at each time of index determination for three such experiments was calculated. Since the determinations in the first of the three experiments were not continued beyond 120 minutes, the average value for 180 minutes was obtained from the last two experiments.

The average distal pigment indices obtained from the three experiments for each time that a determination was made were used to prepare Figure 1(C). It can be seen from this graph that the rate of light-adaptation and the final degree achieved with one-eyed animals are less than that with normal animals. The distal pigment index reached by the one-eyed animals is quite comparable to that seen following the removal of one eye from normal animals in light. It appears, therefore, that both eye-stalks are essential for the normal rate and degree of light-adaptation of each eye under these conditions of illumination.

Influence of a Brief Light Exposure

The extensive light-adaptation in the dark which results from the interruption of a long dark period by a one-minute exposure to light at 250 ft.-c., described elsewhere (Brown, Fingerman and Hines, 1952), suggested the operation of a light-adapting hormone. It appears that an adequate stimulus for secretion of such a substance is a brief, bright, light flash and that the activity of the substance long outlasts the duration of the original brief stimulus. Prawns with a single eyestalk and in darkness responded to a one-minute, 250 ft.-c. flash by substantially less light-adaptation than that seen in normal prawns subjected to a similar stimulus.

It was desired further to determine whether a second substance might be operating in dark-adaptation as postulated by Brown, Fingerman and Hines (1952).

Since a change from darkness to light is evidently an adequate stimulus for the release of the light-adapting hormone it seemed reasonable that a change from light to darkness would be a normal stimulus causing secretion of a dark-adapting hormone. In order to test this possibility an experiment was designed in which responses to a brief light flash were compared following different durations of dark periods.

Palaemonetes were taken at random from the stock supply and distributed among six groups of five white enamelled pans. Approximately 12 animals were put into each pan. The first group of animals constituted the control group. Distal pig-

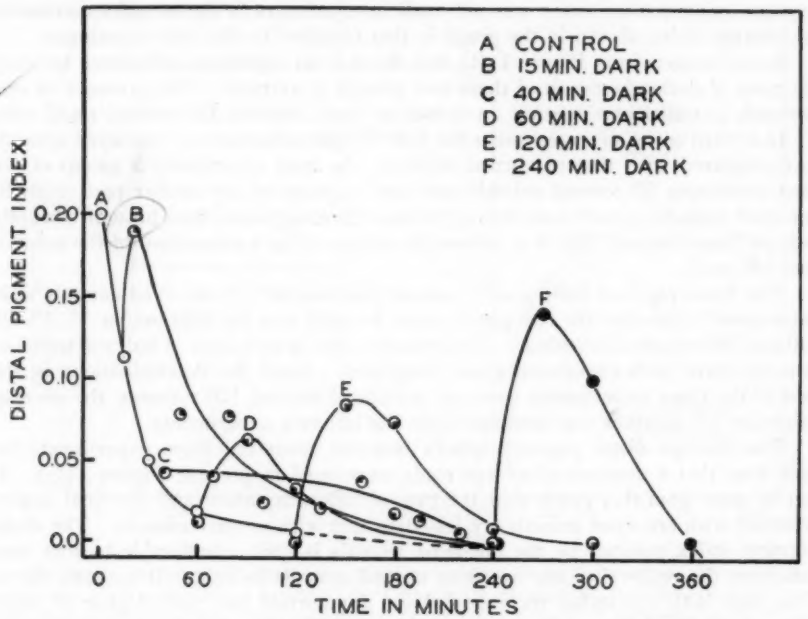

FIGURE 2. Responses of the distal pigment to a one-minute, 250 ft.-c. light flash after different intervals in darkness.

ment indices of 10 animals from one of the pans of this group were determined at 9 A.M. and the remaining containers were placed immediately in darkness. The course of dark-adaptation was followed by removing a pan from darkness and determining distal pigment indices of 10 animals after 15, 30, 60 and 120 minutes.

The remaining five groups of pans were also placed in darkness at approximately 9 A.M. At 15 minutes one group of five pans was subjected to a one-minute, 250 ft.-c., flash of light. Immediately following the flash the distal pigment indices of 10 animals from one pan were determined. The course of dark-adaptation of this group was followed by determining distal pigment indices of 10 animals after 30, 60, 120, and 180 minutes. Similarly, of the other groups, one was dark-

adapted for 40 minutes, one for 60 minutes, one for 120 minutes and the last for 240 minutes. At the end of each of these periods the group of animals was treated in a similar manner to that described for the 15-minute group.

In all the groups for each time that a determination was made an average distal pigment index of the 10 animals was determined. These average indices were used to prepare Figure 2 where the distal pigment index is plotted against time in minutes. In Figure 2 zero time represents that time at which all groups were put into darkness for the first time.

It is apparent from this figure that both the amount of light-adaptation induced by the one-minute flash and the rate of subsequent re-dark-adaptation are influenced by the duration of the dark period. By examination of curves C, D, E, and F, representing 40, 60, 120 and 240 minutes of exposure to darkness prior to the light flash, it can be seen that the longer the period in darkness the greater the extent of light-adaptation resulting from the flash and the more rapid the subsequent rate of dark-adaptation. However, it can also be seen that 15 minutes of exposure to darkness is not sufficiently long to yield a result which is entirely consistent with this generalization. This group light-adapted during the first 15 minutes following the flash to a state almost equivalent to the fully light-adapted state shown by all groups when they were removed from the aquaria at the beginning of the experiment, as illustrated by the initial index of the control group. The rate of dark-adaptation was, however, somewhat less than that shown by the controls.

These results strongly support the hypothesis of the operation of two hormones, one inducing light-, and the other, dark-adaptation. Since greater degrees of light-adaptation result from a one-minute flash of light as the animals are left longer in darkness, it would appear that the ability to secrete the light-adapting principle increases, at least for a few hours, in darkness. On the other hand, the increase in the rate of dark-adaptation with increasing time in darkness can be explained in terms either of an increase in ability to secrete the dark-adapting principle in response to return to darkness or to the presence of a higher titer of this material in the blood for the normal maintenance of the dark-adapted state.

Injection Experiments

In an attempt to obtain some information regarding the sources of the substances which function in the light- and dark-adaptation of *Palaemonetes* distal pigment, experiments were designed in which extracts of eyestalks and of central nervous organs were injected into animals under various conditions.

The first type of experiment consisted of injecting extracts of eyestalks of *Palaemonetes* into animals which had been previously dark-adapted for a minimum of three hours. These animals were taken at random from the stock supply and five were placed in each of four white enamelled pans partially filled with sea water. The extract was prepared by triturating freshly removed eyestalks in a sufficient quantity of sea water to yield such a final concentration that each animal, receiving an injection of 0.02 ml., received the equivalent of one eyestalk. The extracts were centrifuged. The animals were injected in the dark-room under a red photographic light which had previously been tested and shown to produce no light-adaptation. At 30, 60, 120 and 240 minutes one pan of animals was removed from the darkroom and the distal pigment indices of the five animals determined. An

average distal pigment index was calculated for each time that a reading was made. Two experiments of this sort were performed and the results averaged. The averages of the two experiments were used to prepare Figure 3.

It can be seen from Figure 3 that the injection of eyestalk extract produces light-adaptation comparable to that produced by the interruption of a dark period by a one-minute, 250 ft.-c. flash of light. The maximum degree of light-adaptation achieved occurs in 30–60 minutes following injection. The dark-adaptation which followed was not quite complete at the end of 180 minutes.

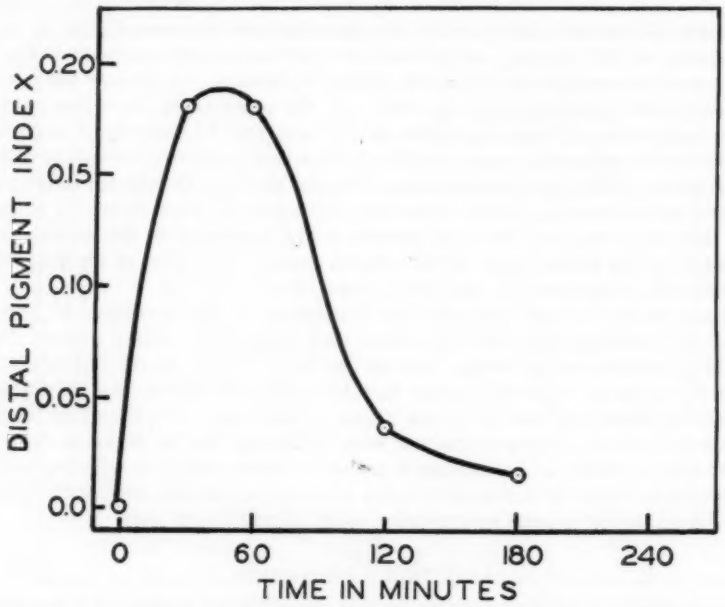

FIGURE 3. Response of dark-adapted prawns in darkness to injection of an extract of eyestalks.

Preliminary experiments in which sea-water extracts of various central nervous organs were injected into similarly dark-adapted *Palaemonetes* yielded inconsistent results. In some cases no light-adaptation was obtained while in others distal pigment indices of the order of 0.03 were obtained, suggesting the presence of light-adapting substance in nervous tissue.

Another method of assay of the extracts was used. A study was made of the influence of injection of extracts of eyestalks and of central nervous organs on the response of dark-adapted prawns to a brief exposure to bright light. For each experiment *Palaemonetes* were taken from the stock supply and distributed among three groups of five pans with five animals in each pan. These animals were all placed in the darkroom overnight and then about 6 the next morning brought abruptly into an illumination of 250 ft.-c. for a period of 20 minutes. During this

period of light exposure one group received injection of an extract of the brain, connectives and ventral cord. The second group received extract of eyestalk, and the third received only sea water. Each animal was given a dose of 0.02 ml.

The extracts of the central nervous system were prepared by dissecting the brain, circumesophageal connectives, and thoracic and abdominal cords from normal light-adapted animals and extracting them in sea water in such an amount that an animal receiving a dose of 0.02 ml. received the equivalent of half a total nervous system. The extract was centrifuged. The extract of the eyestalks was prepared as previously described, such that each animal received one eyestalk or half the equivalent of the complement of a normal animal.

Two experiments of this sort were performed. In the first, distal pigment indices of five animals in one of the pans were determined immediately following the exposure to light while the rest of the containers were placed in the darkroom. Successive determinations of distal pigment indices were made at 30, 60, 120 and 180 minutes, using a different pan of animals for each determination. In the second experiment determinations of the distal pigment indices were made at 30, 60, 120, 180 and 240 minutes from the time the pans were returned to darkness. Averages of the distal pigment indices of five animals for each time that a determination was made were calculated and average values for the two experiments were obtained. Since the initial and 240-minute determinations were made in only one of the two experiments, the average obtained in one experiment was used as the definitive one.

The average distal pigment indices for the two experiments were used to prepare Figure 4. It can be seen from this figure that, compared with the sea-water controls, extracts of the central nervous system as well as those of the eyestalks supplement the amount of light-adaptation which occurs as a result of the exposure to light. Furthermore, there is a significant increase in the subsequent rate of dark-adaptation over that seen for the controls. The extracts, therefore, appeared to contain both light- and dark-adapting principles.

In an effort to determine whether the two hormones are differentially distributed within the central nervous system further experiments were designed in which extracts of various parts of the nervous system were compared with extracts of the eyestalks. In preliminary experiments of this sort it was found that extracts of the abdominal cord, thoracic cord, circumesophageal connectives and the brain all behaved qualitatively like an extract of the total nervous system. However, this seemed not to be true for extracts of the tritocerebral commissure.

In two experiments sea water extracts of eyestalks, tritocerebral commissures, and sea water were injected into three groups of overnight-dark-adapted *Palaemonetes* while they received a ten-minute exposure to an illumination of 250 ft.-c. at about 6 A.M. The extract of eyestalks was of such concentration that an animal receiving a dose of 0.02 ml. received the equivalent of half an eyestalk or a quarter the equivalent of the complement of a normal animal. The extract of tritocerebral commissure was prepared by removing the brain and circumesophageal connectives from several animals in such a manner that the tritocerebral commissures were not damaged. As they were removed, these organs were placed in sea water in a Syracuse watch glass until the desired number had been obtained. The commissures were then carefully removed by severing them with a scalpel at their junctions with the circumesophageal connectives. They were then transferred to a microscope slide

with a minimum of water and triturated as completely as possible with a small glass pestle with sea water to yield a concentration such that an animal receiving the usual dose of 0.02 ml. would receive the equivalent of ¼ of a commissure.

Each extract, including sea water as a control, was injected into five groups of 10 animals. After the light exposure and simultaneous injection of the three groups the animals were returned to the darkroom and the course of dark-adaptation was followed by making distal pigment index determinations for 10 animals of each group at 30, 60, 90, and 120 minutes from the beginning of the light flash.

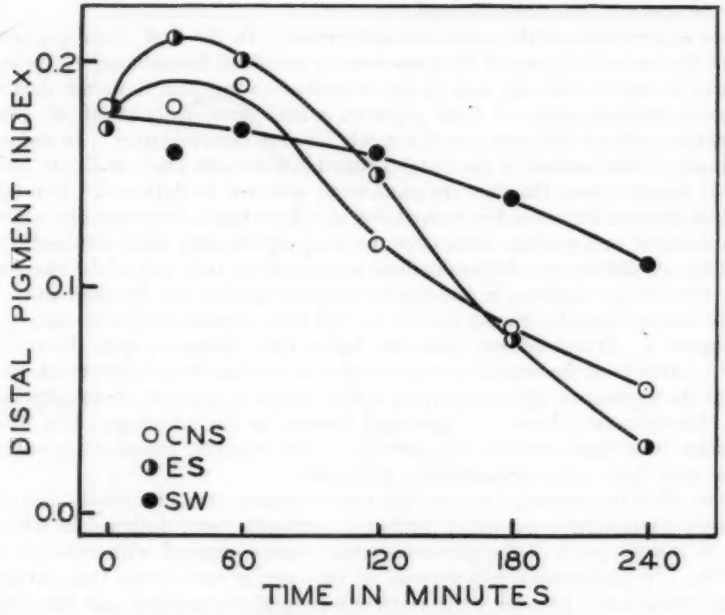

FIGURE 4. Comparison of the light- and re-dark-adaptational responses of prawns overnight in darkness to a simultaneous 250 ft.-c. light stimulus and injection of extracts or sea water. CNS—central nervous system; ES—eyestalk; SW—sea water.

It can be seen from Figure 5 that the injection of extract of tritocerebral commissure during the light flash resulted in a light-adaptational response which was significantly less than that achieved by the animals receiving only sea water. The animals receiving extract of eyestalks responded in a manner essentially similar to those in the previous experiments. The extent of light-adaptation was substantially greater than that shown by the sea water controls. These results suggested that in the tritocerebral commissure the dark-adapting hormone was present without the light-adapting one.

In order to establish further the character of the influence of the extract of the tritocerebral commissures a final experiment was designed. This one continued for a period of time long enough to follow re-dark-adaptation. In this experiment

three groups of three pans containing 10 animals in each were given a one-minute, 250 ft.-c. flash of light at 6 A.M. and then returned to the darkroom. At the end of an hour in the dark when the animals were expected to be maximally light-adapted as a result of the flash, they were brought into 250 ft.-c. illumination again for ten minutes during which time one group was injected with extract of tritocerebral commissure, the second group with an extract of eyestalk, and the third group with sea water as a control. The extracts were prepared in the usual manner. Following injection the three groups of pans were returned to the darkroom and distal pigment indices of 10 animals in each group were determined at 30, 60, 150, 210 and 270

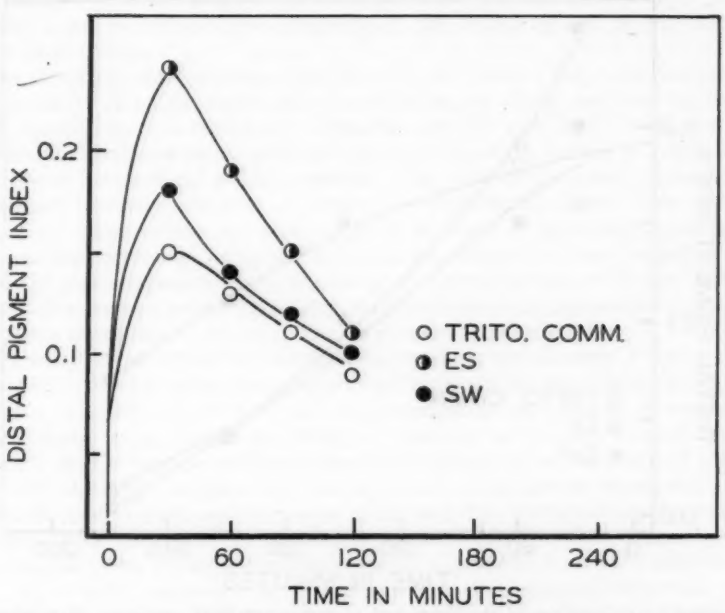

FIGURE 5. Comparison of the light- and re-dark-adaptational responses of prawns overnight in darkness to a simultaneous 250 ft.-c. light stimulus and injection of extracts or sea water. Trito. Comm.—tritocerebral commissure; ES—eyestalk; SW—sea water.

minutes following the beginning of the second light period. Since there were only three containers of animals in each group, each container was returned to the darkroom after each distal pigment index determination so that it could be used for another determination. Thus, container 1 was used for the 30-minute and the 210-minute determination while container 2 was used for the 60-minute and the 270-minute determination. It had previously been determined that the brief exposure to the microscope light during a determination for animals in this experimental state was not significantly effective in inducing light-adaptation. This is substantiated also by the response of the control group of this experiment.

Averages of the distal pigment indices of the 10 animals for each time that a determination was made were calculated and plotted in Figure 6. Zero time in this

figure indicates the beginning of the second light period. The probable index for all three groups at this time was estimated by extrapolation of the control curve.

It can be seen from Figure 6 that the influences of the extracts of tritocerebral commissure and eyestalks are qualitatively the same as in the previous experiment. Tritocerebral commissure depresses while eyestalk supplements the degree of light-adaptation following equal light exposure. However, the rate of dark-adaptation of the animals receiving extract of tritocerebral commissure is substantially the same as for those receiving extracts of eyestalk, and, furthermore, this rate is much

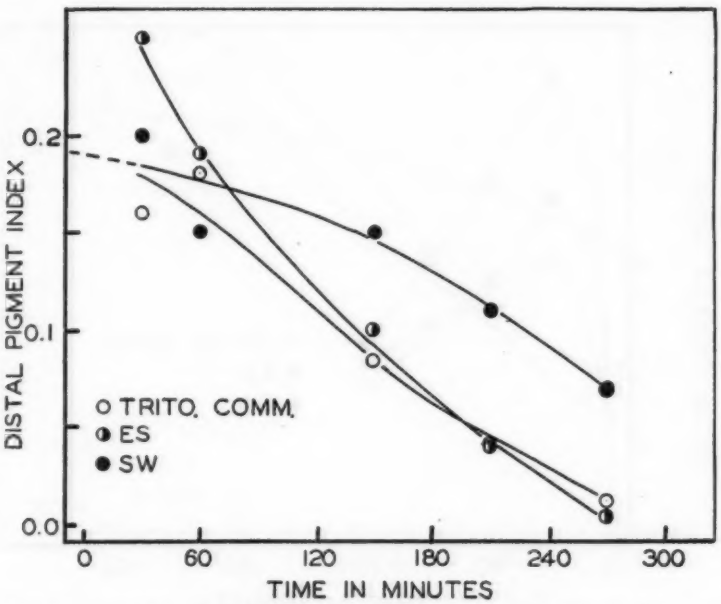

FIGURE 6. Comparison of the light- and re-dark-adaptational responses of prawns overnight in darkness to a simultaneous 250 ft.-c. light stimulus and injection of extracts or sea water. Trito. Comm.—tritocerebral commissure; ES—eyestalk; SW—sea water.

greater than that shown by the sea-water controls. Dark-adaptation of the two experimental groups was essentially complete in 270 minutes while the sea-water controls were still significantly light-adapted (distal pigment index of 0.068) at this time. It appears, therefore, that the tritocerebral commissure contains the dark-adapting principle but no significant amount of the light-adapting principle, while the eyestalks contain both principles.

DISCUSSION

The experiments involving the comparison of responses of animals with a single eye and eyestalk with those of normal animals clearly indicated that both eyestalks

are essential to the normal regulation of the retinal pigment of a single eye. This phenomenon appears to be reasonably interpreted, if taken as an isolated observation, in terms either of (1) loss of one of the two major photoreceptors of the organism or (2) loss of an eyestalk source of an endocrine factor concerned in light-adaptation. In terms of the former, there would be expected to be a reduction in the number of afferent pathways activated by light and consequently of impulses passing into the central nervous system. There could conceivably, therefore, be a reduced excitation of any endocrine gland located anywhere in the body in response to any given intensity of illumination. Although this possibility is a real one, it is rendered less probable by the observation that removal of one eyestalk and eye does not significantly alter the rate of dark-adaptation of the remaining eye in response to a light-to-dark change.

The alternative interpretation, that the removal of one of the stalks has removed a major source of a light-adapting hormone, is equally likely, and this one has the added support that it is completely consistent with the view held for several years that the sinus glands of the eyestalks are the most important sources of a light-adapting hormone for the distal retinal pigment. This view is also given support by the experiments involving injection of extracts reported in this paper. The eyestalks are far more effective than any other organ of the body in light-adapting action.

By similar reasoning, using the failure of the removal of one eyestalk to alter the rate of dark-adaptation of the remaining eye, there is support for the view that the eyestalks are not, relatively, as important sources of a dark-adapting principle. This also is strengthened by the results of injection experiments.

The work reported here has substantiated earlier work (Brown, Fingerman and Hines, 1952) that following one complete discharge of light-adapting hormone, some hours are necessary before there is a regeneration of the capacity to respond as strongly again to an equivalent stimulus. Here it seems quite evident that the endocrine sources become recharged through synthesis or accumulation of the hormone and discharge it again only in response to appropriate stimulation. This conclusion appears quite secure in view of the fact that the light-adapting hormone exercises a dominance over the dark-adapting one. That is, when both are present in substantial titers, the light-adapting principle appears to exert an action which is only very slightly depressed by the presence of dark-adapting hormone. Only when the conditions are such that there is a reduction in the quantity of light-adapting hormone present can the dark-adapting hormone exert that action which is then a function of its concentration.

On the other hand, the interpretation of the increase over some hours in darkness of the capacity to re-dark-adapt following the light-adaptational response to a one-minute, 250 ft.-c. flash is more difficult because of the aforementioned dominance. There may be either (1) a recharging of the endocrine glands concerned with the production of the dark-adapting hormone which is later discharged in response to the light-to-dark change which terminates the light flash, or (2) there may have been a gradual increase in blood titer of a dark-adapting hormone having such properties that it disappears only slowly from the blood over a period of some hours.

There is still too little information to permit one to reach a decision as to whether the dark-adapting hormone becomes stored in the glands of its origin in darkness.

The results do suggest, however, that there is no accumulation of light-adapting hormone in the sources in animals which are maintained for some hours in constant light at 250 ft.-c. Such animals, immediately following dark-adaptation during which it is presumed there would be no induced liberation of light-adapting hormone, have but little power of responding to a brief light flash by light-adaptation. On the other hand, there is the suggestion that the sources of dark-adapting hormone are provided with accumulated dark-adapting hormone when they are taken from constant illumination or during the day from the laboratory tanks where they have been subjected to the normal daily variation of illumination. Such animals can dark-adapt rapidly. However, immediately following induced dark-adaptation, these animals are unable to respond to a light-to-dark change with other than a very slow rate of re-dark-adaptation of their distal retinal pigment.

As was done by earlier investigators, attempts were made during the course of this work to induce dark-adaptation of the distal retinal pigment in light-adapted animals maintained in light by injection of various extracts. It might be presumed from the work reported herein that extracts of tritocerebral commissures would have yielded such results, but this was not the case. There are two reasonable explanations for such failure. One of these is to be seen in the dominance of the light-adapting hormone and the fact that all of the experiments were performed at intensities of illumination producing complete or nearly complete light-adaptation. At such intensities the state of the pigment would be expected to be determined predominantly by the light-adapting hormone. A second possible explanation is that the regulatory powers of the animals to illumination are such that the animals compensate for any alterations due to the injections by the secretion of more of the dominant light-adapting principle. It is interesting, in this latter connection, that the only condition under which it was possible in this work to demonstrate the presence of a dark-adapting hormone by injection was under the environmental condition of complete darkness. This condition would be expected to call forth maximal reduction in the blood titer of light-adapting hormone, and furthermore this is a condition to which there could not be expected to be any active adjustment.

Finally, it is evident from the experiments which have been described that, at least in darkness, injected light-adapting hormone disappears more rapidly from the blood than does injected dark-adapting hormone.

Summary

1. After removal of one eyestalk the distal pigment of the intact eye of *Palaemonetes* light-adapts more slowly and to a less extent for a given illumination; dark-adaptation is unaffected.

2. The eyestalks are the chief sources of light-adapting hormone; lesser amounts are found in brain, connectives and ventral ganglia.

3. The eyestalks and central nervous organs are sources of dark-adapting hormone. The tritocerebral commissure possesses dark-adapting, but no light-adapting hormone.

4. Both light- and dark-adapting hormones can be elaborated and stored, to be discharged in quantity upon appropriate stimulation.

5. Light-adapting hormone is elaborated and stored during a few hours in darkness; no store appears to be present in prawns kept in light.

6. Dark-adapting hormone appears to be stored in animals in light; following transfer to darkness, the store is depleted but the capacity to re-dark-adapt following response to a brief light flash gradually increases during a few hours in darkness.

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EFFECT OF ADRENAL PREPARATIONS ON TUMOR GROWTH

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Evidence has been accumulating in the literature for an influence of the adrenal cortex on malignant growth. Beck and Diller (1946) and Diller *et al.* (1948) reported definite degenerative changes and decrease in size of transplanted mouse sarcoma 37 following intraperitoneal injections of 0.5 to 1.0 cc. of a potent adrenal extract.

It seemed of interest to us, therefore, to investigate the effects on sarcoma 37 of more extended treatment with adrenal extract and with certain fractions obtainable from the adrenal. The effect of desoxycorticosterone acetate (DCA) was observed for comparison only since this substance is not an adrenal hormone.

Methods

All animals used in this study were adult albino mice of the Carworth Farms strain, 10 to 12 weeks of age. A number of animals bearing sarcoma 37 were obtained from the Lankenau Hospital Research Institute through the courtesy of Dr. Irene Corey Diller. The tumor was kept growing in our own laboratory by transplanation into female mice every 10 to 14 days. This material provided the stock tumor for all experiments. Since sarcoma 37 sometimes shows spontaneous regression, experimental and control groups were always implanted and run simultaneously in the following manner, after the method of Dr. Diller (Diller, 1947). Viable tumors were removed from the host aseptically, divided into fragments and drawn into a number 15 trocar for implantation. Inoculations were made subcutaneously into the ventral surface of the recipient, the skin having previously been defurred and cleaned with 70 per cent alcohol. Transplants were routinely allowed a seven day development period. The tumor-bearing mice were then divided into three groups on the basis of tumor size as determined by palpation, *i.e.*, large, medium and small tumors. When animals for the experimental and control series were chosen, equal numbers were taken from each of these groups. In most cases, except as noted below, they were injected twice daily with the various preparations for periods ranging from 5 to 7 days. The age of the tumors at the time the animals were cancelled was 15–17 days. On the day following the injection period experimental and control groups were killed with ether. After being measured with calipers along two axes, one in a right angled plane to the other, the tumors were carefully removed and the living tissue separated from necrotic areas with the aid of a binocular loupe. The living tissue was weighed in a glass-stoppered weighing bottle either from individuals or from experimental groups. In some cases as noted, dry weight was also determined. The following preparations were used in experiments as designated: 1. Upjohn's whole adrenal extract, 1 cc. = 60 g. tissue; 2. Whole adrenal extract prepared in our own laboratory, 1 cc. = 60 g. tis-

sue; 3. DCA [1] in oil, 1 cc. = 5 mg.; 3a. DAC in 15% alcohol, 1 cc. = 5 mg.; 4. sodium hormone in 10% alcohol prepared in our laboratory by Dr. J. S. Thatcher, 1 cc. = 300 g. tissue; 5. 17-hydroxy-11-dehydrocorticosterone [2] in 10% alcohol, 1 cc. = 0.6 mg.; 6. 11-dehydrocorticosterone [3] in 10% alcohol, 0.6 mg./cc.

Results

Effect of whole adrenal extract

The results with adrenal extract, though variable, were so pronounced as to leave little doubt of its power to inhibit the growth of sarcoma 37 in female mice when adequate dosage was used (see Table I). Thus, one injection of 0.5 cc.

Table I

Effect of whole adrenal extract on tumor growth

Group treatment	No. of mice	Dosage daily, cc.	Wt. of living tumor tissue				Degree of necrosis			
			Wet wt. avg.	% change	Dry wt. avg.	% change	0	+	++	+++
1 Adrenal Ext. A	12 ♀	.1 4×	.151	+104	.122	+198				
Controls	13 ♀		.069		.041					
2 Ext. A	15 ♀	.3 2×	.137	+37	.029	+27				
Controls	15 ♀		.100		.023					
3 Ext. B	17 ♀	.4 2×	.076	−42	.016	−41	0	5	6	6
Controls	20 ♀		.131		.027		1	7	8	1
4 Ext. B	9 ♂	.4 2×	.154	+208	.030	+123				
Controls	8 ♂		.050		.013					
5 Ext. C	7 ♀	*	.262	0			0	3	4	0
C	7 ♀	.1 1×	.213	−18			0	3	2	2
C	7 ♀	.2 1×	.031	−88			0	0	2	5
Controls	7 ♀		.260				0	5	2	0
6 Ext. C	11 ♂	.1 2×	.189	−28			2	3	4	2
Controls	10 ♂		.264				1	2	7	0

*—1 inj. only, 0.5 cc. 5 days before cancellation.
A—our own.
B—Upjohn, lot 1.
C—Upjohn, lot 2.
0—none.
+—slight.
++—moderate.
+++—complete.

(Group 5) five days before cancellation had no effect on tumor growth; 0.1 cc. injected daily for 5 days (Group 6) prior to cancellation caused a negligible (18 per cent) reduction in amount of viable tumor tissue present considering the variability, while 0.2 cc. injected daily for 5 days (Group 6) brought about almost complete disappearance of viable tissue as compared with controls. At the dosage level of 0.2 cc. daily for five days, there was a 28% reduction in amount of viable tumor tissue

[1] Kindly supplied by Roche-Organon, Inc.
[2] Supplied through the courtesy of Dr. E. C. Kendall.
[3] Kindly supplied by Merck and Co.

in male mice (Group 6). This effect is remarkable since in males this tumor has been found notably resistant to adrenal preparations (Diller *et al.*, 1948). Another batch of adrenal extract was found to have no inhibiting effect at daily dosage levels of 0.4 and 0.6 cc., but at a level of 0.8 cc. daily the inhibition of sarcoma 37 in female mice was marked (Table I, Group 3), amounting to 42%. This extract at the same dosage level caused an actual increase in tumor growth in male mice. It is interesting to note that extract seems to reduce tumor size not so much by inhibiting growth as by accentuating the processes leading to necrosis. The data on degree of necrosis show this. In the group of 7 animals treated with 0.2 cc. extract C daily (Group 5), two showed a moderate degree of necrosis (++) while in five necrosis was complete (+++). In the controls of this series two exhibited a moderate degree of necrosis (++) while in five necrosis was very slight (+). Again in Group 3 about the same relationship obtains as to degree of necrosis observed, there being 6 out of 17 tumors completely necrotic (+++) in the extract-treated group while only one tumor out of 20 in the control series showed total necrosis. This observation is further substantiated by tumor measurements on this group at the time of killing. The average measurements on the control tumors were 91 × 127 mm. while those on the experimental group were 98 × 125 mm. or approximately identical. However, on weighing the living tissue from each group, the average value for the treated animals was found to be 0.076 g., 40 per cent below that of the control animals (0.131 g.).

Effect of sodium-retaining substances

Because of the small amount of sodium hormone available, this treatment was tried in only one group of 15 animals (Table II, Group 5). It resulted in a questionably significant reduction (26 per cent) in living tumor tissue when compared to the control group. As in the case of whole adrenal extract the reduction appeared to be due to more rapid progress of necrosis rather than to direct

TABLE II

Effect of sodium-retaining substances on tumor growth

Group treatment	No. of mice	Dosage daily, cc.	Wt. of living tumor tissue				Degree of necrosis			
			Wet wt. avg.	% change	Dry wt. avg.	% change	o	+	++	+++
1 DCA in oil	6 ♀	*	.172	+60			0	2	2	2
Controls	6 ♀		.108				0	1	1	4
2 DCA in oil	13 ♀	.1 4×	.243	+254			10	3	0	0
Controls	13 ♀		.069				9	3	1	0
3 DCA in alcohol	15 ♀	.3 2×	.175	+75	.036	+55	2	6	4	1
Controls	15 ♀		.100		.023		5	7	2	1
4 DCA in oil	11 ♂	.1 2×	.312	+18			2	5	4	0
DCA in alcohol	11 ♂	.1 2×	.272	+3			0	4	6	1
Controls	10 ♂		.264				1	2	7	0
5 Sodium factor	15 ♀	.1 2×	.085	−26	.018	−22	1	4	7	3
Controls	15 ♀		.115		.023		9	3	3	0

* 0.5 cc. subcut., 0.5 cc. intraper. 7 days before cancellation.

TABLE III

Effect of some crystalline gluconeogenic substances on tumor growth

Treatment	No. of mice	Dosage	Wt. of living tumor tissue	
			Wet avg.	% change
17-hydroxy-11-dehydrocorticosterone (cortisone)	7 ♀	1 inj. 300 γ in 10% alc. 5 days before cancellation	.428	+65
11-dehydrocorticosterone	7 ♀	1 inj. 300 γ in 10% alc. 5 days before cancellation	.385	+48
Controls	7 ♀		.260	

inhibition of growth. It will be noted in the table that three sodium hormone-treated animals exhibited complete necrosis (+++) while seven showed a moderate amount (++) and only one animal showed none, while in nine animals of the control group no necrosis was observed and none of the others showed marked necrosis. This rather striking difference in necrosis in favor of the sodium hormone-treated animals further strengthens the questionably significant data on tumor tissue weights. The synthetic sodium-retaining substance, DCA, had the opposite effect on tumor growth. All dosages employed produced marked increases in tumor growth in female animals; in the male insignificant increases were observed (Group 4, Table II). DCA in oil appeared to be a more potent tumor growth stimulant (254 per cent increase) than DCA in alcohol (75 per cent increase), probably because in alcohol the substance is more rapidly absorbed and destroyed. It will be noted (Group 3) that the increase in living tumor tissue is significantly more on the wet weight basis than on that of dry weight. This is to be expected from the known action of desoxycorticosterone acetate in increasing body water generally.

Effect of crystalline gluconeogenic substances

The amounts of crystalline gluconeogenic compounds available at the time these observations were made seriously limited our study of these substances. Seven female mice were injected subcutaneously with 300 gamma each of 17-hydroxy-11-dehydrocorticosterone (cortisone) and an equal number of mice with the same amounts of 11-dehydrocorticosterone. All were cancelled five days later. Both substances definitely increased tumor growth as compared to controls (Table III), and there was no significant difference between them. In the case of the cortisone-treated animals there seemed to be some increase in amount of necrosis over that found in control animals. This might be due to the cortisone treatment or possibly to larger tumors more rapidly outgrowing their blood supply. From the data available there is no means of differentiating these two factors.

DISCUSSION

There is some evidence that a low level of adrenal cortical secretion is associated with tumor growth and that a high level is inhibitory to such growth. Thus, Haven and Asheworth (1950) found steroids in the adrenal markedly decreased in rats bearing Walker tumor 256 even though the gland weights in these animals were

significantly increased, and Dobriner and co-workers (1950) found urinary ketosteroid excretion low in patients with neoplastic disease. Haven *et al.* (1949) observed somewhat increased steroid content of the adrenals of rats in which Walker tumor 256 either did not take, or in which it took and regressed.

Of the direct, inhibitory effect of adrenal grafts, adrenal tissue extracts, synthetic adrenal hormones or synthetic products possessing some properties similar to adrenal hormones, most observations seem to be on lymphoid tumors, or leukemia (Law and Speirs, 1947; Murphy and Sturm, 1944, 1950; Woolley, 1950). These authors all observed at least temporary regression. Their results are in line with the well known observations of involution of the thymus and lymph nodes when additional cortical hormones are administered to normal animals. Of such effects on sarcoma 37, the only evidence of which we are aware aside from our own (Table I) is that of Beck and Diller (1946) and Diller *et al.* (1948) using small amounts (0.5 to 1.5 cc.) of Upjohn's adrenal extract as noted in the introduction. In our hands one injection of 0.5 cc. Upjohn's extract had no effect on growth (Table I, Group 5) as measured by weight of viable tissue five days after the treatment. There are two possible reasons for this difference. First, we were using a different batch of extract and second, we cancelled our animals five days after the injection while Diller *et al.* (1948) noted the greatest incidence of tumor disappearance 17–35 days after treatment.

Our results using cortisone (Table III) are at variance with those reported in the literature in that with sarcoma 37 it produced an actual increase rather than decrease in tumor growth. Heilman and Kendall (1944) working with lymphoid tumors, Higgins *et al.* (1950) using transplanted rhabdomyosarcoma and Burchenal *et al.* (1950) observing leukemic mice obtained results in the direction of inhibition or regression. All of these workers used massive doses of cortisone.

Both large and small doses of DCA (Table II) also stimulated the growth of sarcoma 37. The work of Lipschütz and Zañartu (1942) showed this substance to be anti-fibromatogenic in female guinea pigs treated with estrogens. On the other hand, Kupperman and Greenblatt (1946), working with transplanted sarcoma in rats, obtained results comparable to ours (Table II), namely an enhanced growth of the tumor in animals treated with DCA. It will be noted (Table II, Group 3) that the increase in tumor tissue is considerably greater on the wet (75 per cent) than on the dry weight (55 per cent) basis. This is in line with observations in the literature on other tissues taken from animals treated with DCA (Zuckerman *et al.*, 1950). Although the sodium hormone acts like DCA in causing sodium retention it is unlike this substance in other respects (Thatcher and Hartman, 1946, Hartman *et al.*, 1939). It is interesting to note that the two compounds also differ in their effect on sarcoma 37; DCA caused a marked increase in growth of the tumor while sodium hormone caused a questionably significant reduction in amount of viable tissue present at autopsy and more rapid progress of necrosis than occurred in controls. The sodium hormone used on these animals was not a pure substance. It was purified only to the extent that it contained no gluconeogenic hormone and therefore presumably no compounds with oxygen in the C_{11} position. It is noteworthy that such an adrenal fraction possesses some power to inhibit the growth of sarcoma 37 and to promote necrosis. There is, in the finding of Dobriner *et al.* (1950), that steroids with oxygen in the C_{11} position are *not* decreased in the urine of patients with neoplastic disease while other steroids are, a further suggestion that

other adrenal hormones are more important in this condition than those with gluconeogenic properties. Further investigation of the sodium hormone fraction is planned.

SUMMARY

1. The effects of adrenal extract, sodium hormone, DCA, 17-hydroxy-11-dehydrocorticosterone (cortisone) and 11-dehydrocorticosterone on transplantable mouse sarcoma 37 have been investigated. More than 250 animals were used.

2. Injection of adrenal extract for 5 days caused as high as 88% decrease in viable tissue and marked necrosis of the tumor as compared to controls.

3. Sodium hormone caused a questionably significant decrease in viable tissue (26%) and marked increase in necrosis.

4. DCA increased tumor growth in all doses employed.

5. Both cortisone and 11-dehydrocorticosterone in single relatively small doses (300 γ) enhanced the growth of sarcoma 37.

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THE LARVAL DEVELOPMENT AND ECOLOGY OF THORACOPHELIA MUCRONATA (TREADWELL)

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The Opheliid polychaetes are well known for their localized distribution. Most species are restricted to soils with a relatively narrow range of particle size, some characteristic of fine muds, others of relatively coarse sands. *Thoracophelia mucronata,* like the common species of Ophelia, is found in sands subjected to a relatively heavy wave action, occurring inter-tidally in beaches experiencing fairly heavy surf on the Pacific coast of North America, from Vancouver Island (49° N) to the Punta Banda region, Mexico (31°30′ N). The ecology and life history of such species are particularly interesting, since the larvae, which in all known instances are planktonic, settle on a relatively restricted and unstable substratum.

The development of only one species of Opheliid, *Ophelia bicornis* Savigny, has been worked out in any detail (Wilson, 1948), and although the larval development of *Thoracophelia mucronata* follows a similar plan, there are a number of interesting differences. McConnaughy and Fox (1949) give a brief account of the early development of *Thoracophelia mucronata.* Feeding, and certain aspects of the biochemistry of metabolism, have been studied in this species by Fox, Crane and McConnaughy (1948), and McConnaughy and Fox (1949).

The present study was made over a period of rather less than one year from October, 1950, on the same population studied by Fox and his co-workers at La Jolla, California (32°52′03″ N, 117°15′11″ W).

I am glad of this opportunity to thank Professor Martin W. Johnson, and Professor Dennis L. Fox, of the Scripps Institution of Oceanography, University of California, for many courtesies.

Reproduction and Development

The species is dioecious, males and females occurring in approximately equal numbers. Externally alike, they can be distinguished only by the character of the coelomic germ cells. Coelomic oocytes are colorless, biconvex discs 65 μ in diameter, and 25 μ – 30 μ thick at the center (Fig. 1a, a′). The ripe spermatozoon (Fig. 1b) has a dark acrosome, and a tail about 45 μ in length. As in other polychaetes, the spermatozoa arise from "sperm plates" in the coelom. The worms are apparently atoquous, spawning taking place in or on the surface of the sand.

Fertilizations were easily achieved with really ripe gametes, by slitting open the adults and mixing the eggs and sperm in shallow dishes. Surplus sperm could be washed off, and the larvae which eventually swam to the surface transferred to larger vessels.

Development was extremely rapid (at 15° C. – 18° C.), most of the eggs having reached the 16-cell stage within 2 hours after fertilization. Polocytes are large and

clearly distinguishable during the early stages of cleavage (Fig. 1c, e, f). The blastomeres of the 4-cell stage are approximately equal in size, so that the direction of rotation of the subsequent cleavage could not be determined merely by inspection. After about six hours an equatorial prototroch has developed, and the young larva swims off the bottom soon afterwards (Fig. 1g). An apical tuft of filaments and a single filament slightly dorsal to the vegetal pole are also distinguishable in the swimming trochophore (Fig. 1g). The larvae at this stage are positively phototactic, and are vigorous swimmers. After about five days the larva (Fig. 2a, a′) has reached a length of about 100 μ, and has become distinctly asymmetrical. The prototroch is more prominent, is composed of several rows of cilia, and completely

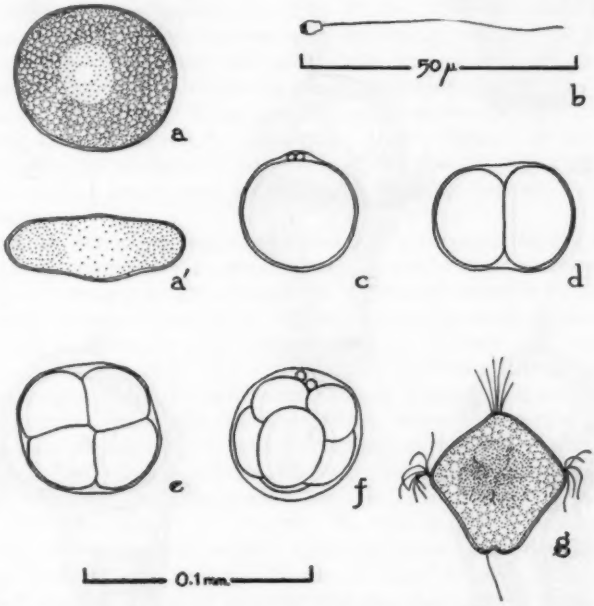

FIGURE 1. Early development. (a), (a′) ripe coelomic oocytes in surface and edge view; (b) spermatozoon; (c) fertilized egg with 2 polocytes; (d) 2-cell stage; (e) 4-cell stage; (f) 8-cell stage; (g) trochophore.

encircles the larva. By this time a telotroch has also appeared, and the mouth and anus have opened into the gut. The anterior apical tuft and the posterior filament remain unchanged until they are lost at metamorphosis.

Throughout the remainder of its planktonic life the larva is markedly asymmetrical, the head region projecting ventrally, forming a large overhanging ciliated lip so that the mouth eventually comes to be directed posteriad, and the stomodeum and the anterior part of the gut are twisted so that the buccal cavity lies slightly to the right, the precursor of the proboscis to the left of the mid-line. Thus at this stage, and in older planktonic larvae, three distinct regions of the gut may be identified:

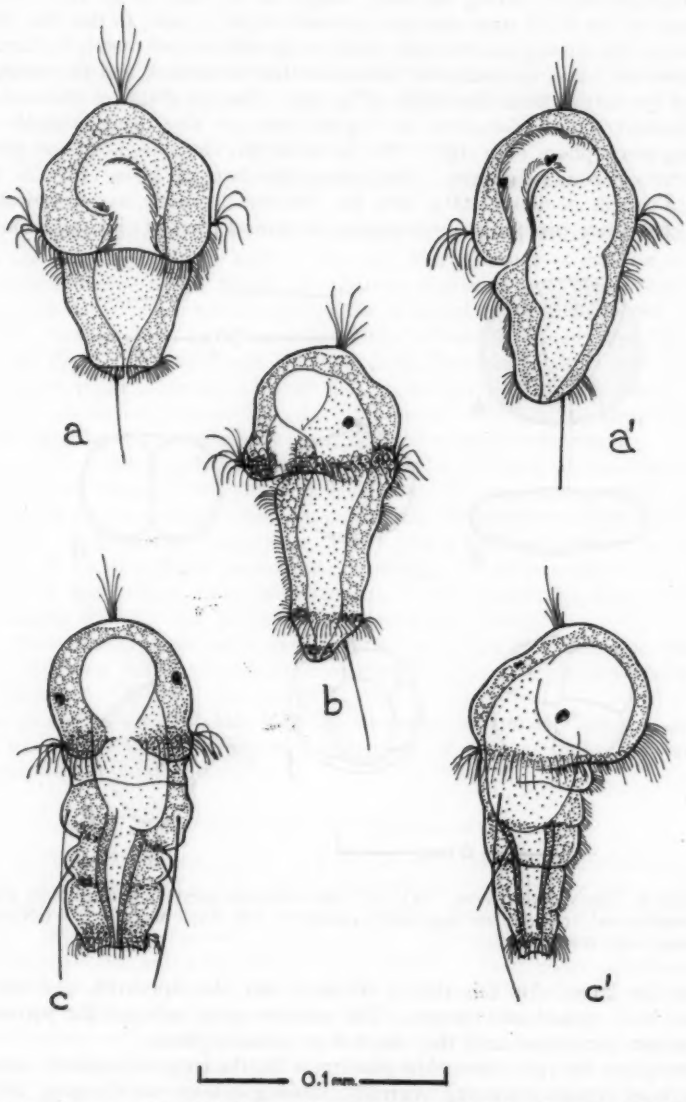

FIGURE 2. Larvae. (a), (a') 5 days old, ventral view, and view from the left side; (b) 7 days old, view from the left side; (c), (c') 9 days old, ventral view, and view from the right side.

(1) a ciliated channel opening into (2) a large non-glandular sac, the precursor of the eversible proboscis, and (3) a straight glandular part leading to the anus. Because of the rapid growth of the anterior part of the gut, it becomes not only bent and slightly twisted, but pushed forwards into the prostomium. This condition is apparently only temporary, since young bottom stages (Fig. 3a) show a relatively straight tube leading from mouth to anus.

As will be seen from Figures 2a′, b, c′, additional tufts or short bands of cilia arise from the ventral region of the larva, and in many larvae form a virtually continuous midventral ciliary band. One pair of rather irregular eye-spots has appeared by the fifth day after fertilization, and in later larvae, three or even four eyes may be seen. By the ninth day two chaetigerous segments have been formed, each at first bearing a single chaeta on either side (Fig. 2c, c′). The two pairs of chaetae appear in rapid succession, the anterior pair projecting well beyond the tip of the pygidium when the chaetae are held against the body while swimming. The first chaetae always appear to be simple capillaries, but winged bristles (Fig. 3b) commonly occur in later larvae. When the larva is nine or ten days old, transverse folds in the cuticle appear between the segments. A very definite division appears between the mouth and prototroch, and the first chaetigerous segment. This region just anterior to this groove may represent the first true segment; this problem is discussed below.

By the tenth day slight projections from the corners of the pygidium may be seen, apparently related to the cement glands which by their secretions enable the larva to adhere strongly to the substratum. Although attempts to rear larvae beyond this stage have not been successful, this almost certainly represents the stage of development at which the larva settles out of the plankton and becomes bottom-living. The apical tuft of filaments and the terminal filament are lost, while the pygidial cement glands aid the larva to maintain its position on a suitable substratum when once found. The planktonic life of these larvae is, therefore, probably not more than about ten days. The head is distinctly darker than the rest of the body at this stage. The trilobed proboscis characteristic of the adult is already differentiated in a larva with ten chaetigerous segments, and is eversible by the time the larva has attained a length of 1.0 mm. At this stage the prostomium is still rounded (Fig. 3a) and similar to other Opheliid larvae which have been described, and it is not until the larva is about 2.0 mm. in length that the prostomium has become pointed, and has coalesced with the first two chaetigerous segments (Fig. 4). These segments become cut off from the thoracic region by a transverse septum, the septa between the succeeding segments being absent or ill defined. The sac which opens dorsally through this transverse septum into the anterior or "head" coelom and which extends back to the fifth or sixth chaetigerous segment is already well defined in a young worm 2.5 mm. in length. The whole apparatus is probably an adaptation for burrowing, as McConnaughy and Fox suggest.

The four anal papillae associated with the cement glands (Fig. 3a) are posterior and ventral to the anus. None of these papillae represents the single large ventral cirrus of the adult (as McConnaughy and Fox have stated), since in young worms between two and four millimeters in length the four larval papillae may clearly be seen on the tip of the adult cirrus as it grows out from the pygidium (Fig. 3d). The dorsal cirri are similarly derived from the pygidium, although the last few

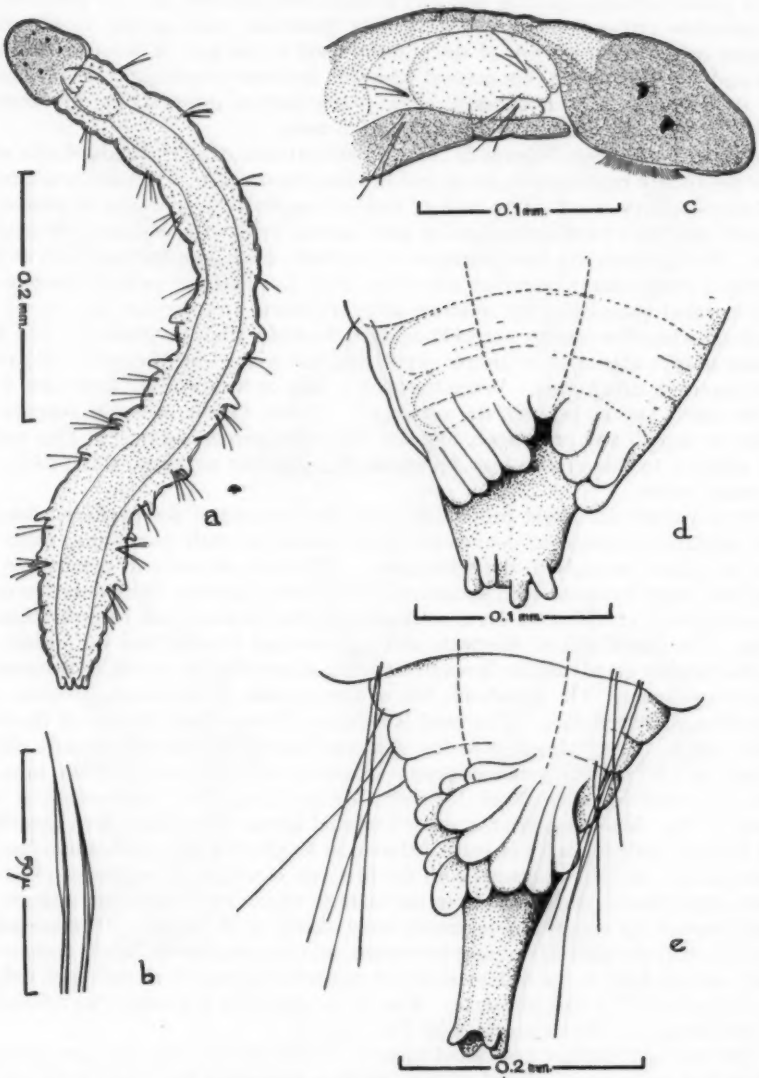

FIGURE 3. (a) Young bottom stage; (b) winged chaetae; (c) side view of the head region of a bottom larva 0.6 mm. long; development of the pygidial region; (d) condition in a young worm with about 26 chaetigerous segments; (e) in a young worm with 36 chaetigerous segments (4 mm. long).

chaetigerous segments become closely associated with it, the chaetae of these segments eventually projecting beyond the anus (Fig. 3e).

Discussion

It is interesting to compare the larval development of *Thoracophelia mucronata* with that of *Ophelia bicornis* as described by Wilson:

Ophelia bicornis	*Thoracophelia mucronata*
Ripe females metallic green in color; males white or pale cream	Ripe males and females similar, the adults containing so much hemoglobin that sexually mature individuals appear only slightly whiter than immature or recently spawned worms
Mature coelomic oocytes oval plates 150 $\mu \times$ 130 μ	Mature coelomic oocytes biconvex discs 65 $\mu \times$ 25 μ
Trochophores produced within 24 hours	Trochophores produced within 6 hours

Common features:

Trochophore rounded or slightly conical with an apical tuft, and a single filament projecting from the vegetal pole.

Larva at the time of settling with 2–3 chaetigerous segments; the head rather darker than the rest of the body, and the pygidium with adhesive papillae. Eyespots rather irregular in shape and number. Winged chaetae present.

The eggs are distinctly smaller than those of *Ophelia bicornis* described by Wilson, and this, together with the higher temperatures under which the *Thoracophelia mucronata* normally develops, accounts for the more rapid development in the early stages. Even so, the larvae of *Thoracophelia mucronata* do show a certain acceleration in the development of some structures as compared with *Ophelia bicornis*. The structure of the adult *Thoracophelia* is more specialized than *Ophelia* in the possession of an anterior "head coelom" in relation to burrowing, separated as its name implies from an aseptate "thoracic" region. The proboscis is somewhat precocious in its development, the anterior region of the gut becoming pushed forwards and coiled almost within the prostomial region as already described. Owing to surf action on the shores on which the larvae settle, the early development of burrowing structures would probably be of advantage. The shape of the curve in Figure 5 may also be interpreted as a tendency for a relatively rapid delimitation of segments early in development. It will be seen (Fig. 5) that a *Thoracophelia mucronata* larva has twenty-five chaetigerous segments when 2.0 mm. in length, while *Ophelia bicornis* larvae do not possess this number of segments until they have reached a length of 5.0 mm. By the time young *Thoracophelia mucronata* have reached a length of 5.0 mm. the number of segments usually found in adults (38) has been attained, although old worms may be ten times this length.

Wilson describes in *Ophelia bicornis* the fusion of the first segment with the prostomium, though remaining separated by a groove. The existence of this segment is not clear in *Thoracophelia mucronata*, and is certainly not recognizable in later larvae (Fig. 3a). However, in larvae with two chaetigerous segments there is a clear demarcation between the mouth opening and the first chaetigerous segment (Fig. 2c). It is suggested that this region between the mouth and the first

chaetigerous segment represents the first true segment, which in *Ophelia bicornis* is achaetous but distinct, but which in *Thoracophelia mucronata* becomes almost obliterated. That this is likely is shown by the fact that the foregut does develop so far forward as already described, and that the precursor of the eversible proboscis does at first lie anterior to the first chaetigerous segment. Since in most polychaetes the proboscis arises almost entirely within the first segment, it is reasonable to assume that the first chaetigerous segment of *Thoracophelia mucronata* represents the

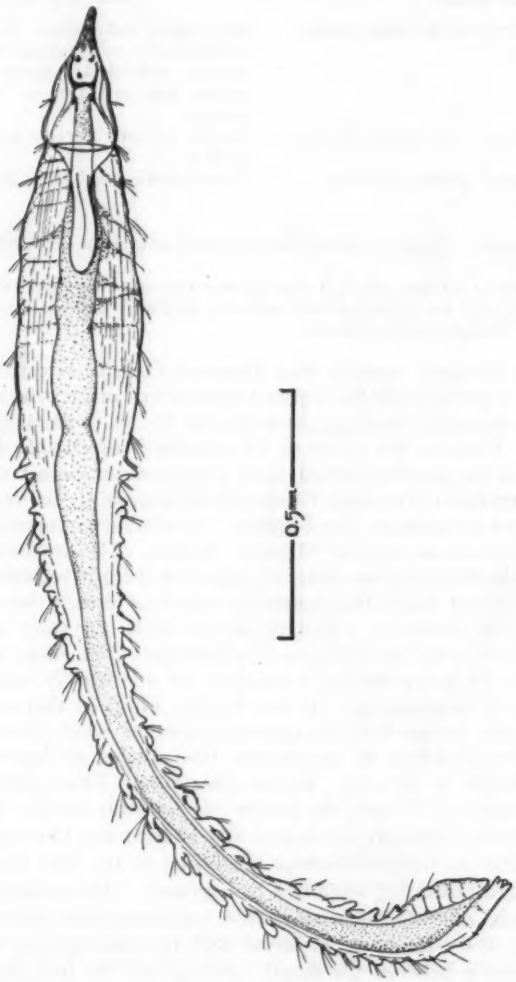

FIGURE 4. Young *Thoracophelia mucronata*

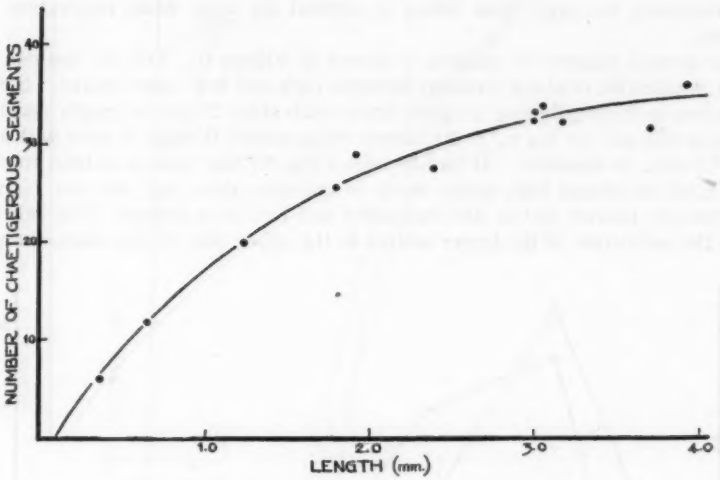

FIGURE 5. Relation of length to number of chaetigerous segments.

second segment, and is homologous with the first chaetigerous segment of *Ophelia bicornis*.

ECOLOGY

The breeding period of *Thoracophelia mucronata* is prolonged during the summer, probably owing indirectly to the equable and relatively high temperatures experienced throughout the year (the temperature of the surface water at high tides varying from a summer maximum of about 22° C. to a winter minimum of about 13° C.). Consequently, the mean length of the worms comprising the population did not vary appreciably from month to month.

Another factor to be considered is the stirring of the surface sand by the surf. Large worms tend to burrow down under such conditions (McConnaughy and Fox, 1949), but a certain proportion are moved after each tide. After rough seas whole areas of the shore normally heavily populated will be depleted of individuals which will be found in immense numbers in channels and pockets where the current loses speed or changes direction. Thus, in spite of the tendency of the larger worms to burrow down, there is an efficient shuffling of the population from time to time, and it is difficult to decide how far the distribution of the worms in the beach is due to purely mechanical agencies. The large amount of hemoglobin which they contain probably acts as an oxygen-store during the periods when the worms burrow deeper to avoid being swept away. When uncovered by the tide, the worms burrow upwards, a small hole appearing on the surface above each worm. This habit is probably respiratory in function, since the worms are usually found head downwards under such conditions, the rectum being used as a kind of lung. The great development of sensory papillae around the anus may thus be understood. The elongate chaetae of the most posterior segments may play some part in breaking the surface

and preventing the sand from falling in around the anus while respiration is in progress.

The general pattern of zonation is shown in Figure 6. The six stations were spaced at intervals of about 6 meters between high and low water marks. Samples were taken each month using a square frame with sides 25 cm. in length (enclosing an area of 625 cm.^{2} or $\frac{1}{16}$ m.^{2}), the sample being passed through a sieve with round holes 0.5 mm. in diameter. It will be seen (Fig. 6) that worms extend from low water mark to almost high water mark of ordinary tides, and are not restricted to a relatively narrow belt as McConnaughy and Fox have stated. This belief was due to the restriction of the larger worms to the upper part of the shore, the popu-

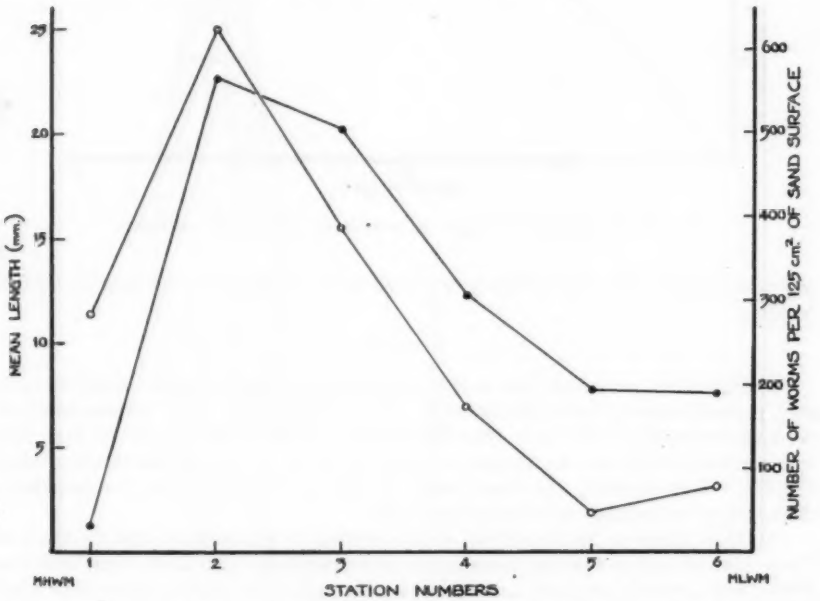

FIGURE 6. Zonation of *Thoracophelia mucronata* at La Jolla. Filled circles = number; empty circles = length.

lation being most dense just above mid-tide level, while the younger worms—which are usually overlooked in the sand owing to their transparency—are restricted almost entirely to the lower part of the shore. A clear division is apparent between the size distribution of worms taken above the mid-tide level where a certain degree of drying out takes place at low tide, and below mid-tide level where the sand remains at or near saturation point (Fig. 7). Two explanations of this division are possible. The larvae may settle over the whole intertidal zone but those above mid-tide level either do not survive owing to the rise in temperature or drying out of the surface sand, or are carried down the beach by the surf. The larger worms are not washed out because of their greater size and weight and ability to burrow be-

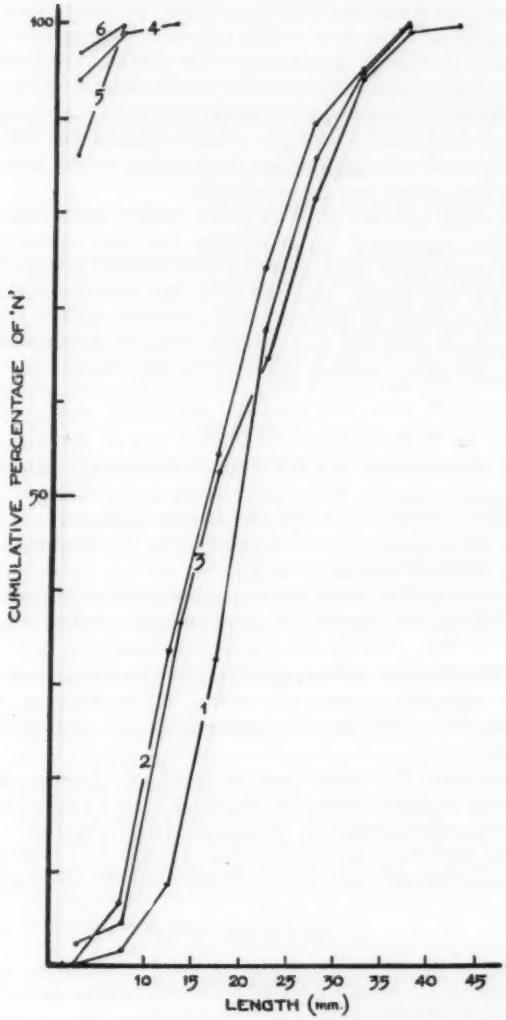

FIGURE 7. Size-distribution of worms at six stations from just below high water mark (1) to just above low water mark (6). Station numbers as in Figure 6.

neath the stirred layer. Secondly, the larvae may settle only below mid-tide level and later migrate upshore. However, it is clear that the optimal zone for the adult worms is appreciably higher than that for the younger stages, but the reason for the relatively sharp division in the mean length of worms from the two zones is not known.

Although there is no doubt that large worms may be redistributed by wave action, under normal conditions there is a certain degree of aggregation as in most polychaetes. The mean density of population at the optimal level was about 10,000–11,000/m.^{2}, but within an area of two or three square meters 625 cm.^{2} samples might contain between 500 and 900 individuals (8000–14,000/m.^{2}, approximately). Since fully grown worms weigh about 0.04 gm. (McConnaughy and Fox, 1949) and are continually passing sand through the gut, their effect on the mechanical structure of beaches where they occur may be appreciable.

Owing to the large amount of hemoglobin which these worms possess, their food-value for other organisms is considerable, and they constitute an important item in the diet of many shore birds, especially the marbled godwit *Limosa fedoa*.

From a study of the figures obtained from the samples made throughout the year, and from observations made under laboratory conditions, it seems likely that these worms take more than one year to mature, and it is probable that each individual lives for a few years, possibly spawning several times.

Summary

1. The larval development of *Thoracophelia mucronata* follows the same pattern as that of *Ophelia bicornis*, but is much more rapid, the eggs being less than a quarter of the volume of those of *O. bicornis* when mature.

2. The larval development shows differences in the formation of the anterior segments and the anterior regions of the gut.

3. Larvae of both species show the development of the adhesive papillae at the time of settling, and this is regarded as an adaption to settling on an unstable substratum.

4. The great development of hemoglobin in *Thoracophelia mucronata* may be interpreted as an adaptation against surf action, the hemoglobin functioning as an oxygen-store when the stirring of the surface layers of sand causes the worms to burrow more deeply.

5. Attention is drawn to the development of sensory papillae, and the elongated chaetae of the most posterior segments in relation to rectal respiration which is carried on near the surface when the sand is not being disturbed.

6. The optimal tidal level for adult worms is above that of the younger stages; the relation between zonation and the size of the worms is discussed.

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IRON ASSIMILATION BY MARINE DIATOMS[1]

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The relationship of marine productivity to the availability of iron has been discussed by Gran (1933), who suggested that organically combined iron introduced into coastal waters from terrestrial run-off is responsible for phytoplankton abundances. He substantiated his argument by demonstrating that the growth of the neritic diatom *Skeletonema costatum* was stimulated by soil extract containing 0.2 micromole of iron per liter, although the iron bound into oxyhemoglobin molecules had no effect. Hopkins (1930) has indicated that ionic iron is necessary for the growth of *Chlorella*, and Rodhe (1948) defines the available iron for utilization in culture by fresh water algae as that which has been stabilized by combination with citrate.

Harvey (1937a) has demonstrated that diatoms are able to assimilate ferric hydroxide or ferric phosphate in colloidal or particulate form and to utilize it in their growth. He has further shown, using the calculated saturation concentrations of ferric and ferrous ions in marine waters, that diatoms accumulate 10,000 times more iron than could possibly be obtained from diffusion of iron ions from the environmental waters. His treatment considered a stationary spherical living cell with respect to the environment, whereas a falling diatom would encounter and take up somewhat more iron.

Continuing an investigation of the minimal quantities of mineral nutrients needed for optimal growth of the marine diatom *Asterionella japonica* (Goldberg, Walker and Whisenand, 1951), an assessment of iron uptake presents the following questions. What is the minimal content of iron per cell needed for further division? What constitutes available iron? Finally, inasmuch as the concentration of iron in marine waters has been found to be highly variable (Cooper, 1948), can iron content be one of many possible parameters in the nutrient index of the productivity equation of Riley, Stommel and Bumpus (1949)?

Culture Techniques

The culture techniques used in the following experiments, unless otherwise indicated, have been described previously in the work on phosphate uptake (Goldberg, Walker and Whisenand, 1951). To maintain symmetry with respect to these investigations the same culture of *Asterionella japonica*, which was isolated from the plankton samples in the winter of 1950, was used. The initial phosphate level was adjusted to 2.5 to 3.0 micromoles of phosphate per liter.

[1] Contribution of the Scripps Institution of Oceanography, New Series No. 572.

Iron Assay

The iron determinations were made either by the colorimetric method of Buch (1942) or through the use of radioactive iron. In the former procedure the extinction of the alpha-alpha' bipyridyl complex of iron was measured at 522 mμ in 100 mm. cells in a Beckman Model DU Spectrophotometer. The method determines both ferric and ferrous iron, but only a portion of the organically bound or particulate iron.

Iron [2] containing the radioactive isotopes Fe^{55}, which decays by K capture, and Fe^{59}, a beta and gamma emitter, was employed as a tracer for this element. The radio iron was plated from aliquots of sea water samples onto 10 cm.^{2} copper planchets following the procedure of Ross and Chapin (1942). As assayed under an end-window Geiger-Muller tube with a window thickness of 1.8 mg./cm.^{2}, the specific activity was 2500 counts per minute per micromole.

Iron Partition in Culture Bottles

In the first experiments with radioactive iron it was found that 80 to 90 per cent of the iron added initially as the ferric citrate complex was adsorbed within one day to the walls of the culture bottle. To eliminate this adsorption, the culture bottles were coated with "Desicote," a polymer of silicon (manufactured by Beckman Instruments, Inc., South Pasadena, California). The coated bottles had no adverse effect upon diatom growth, as was evident from control runs in both treated and untreated bottles. The bottles retained their coating for periods up to one month. At the end of an experiment the bottles were washed with a detergent and thoroughly rinsed with distilled water.

Radioactive ferric citrate, freshly prepared, was introduced into sea water of pH 8.00 to give an iron concentration of 2.2 micromoles per liter. Aliquots of 10 ml. of the solution were centrifuged for thirty minutes and the supernatant liquid assayed radiometrically for iron. The results are listed in Table I. It is observed that hydrolysis of iron citrate occurs to a considerable extent in a period of four days.

As is noted in the table, the decomposition was noticeably accelerated both by light and by stirring. The light effect is probably similar to that reported by Peltz and Lynn (1938) who found that iron citrate complex was partially decomposed in sunlight to CO_2 and a soluble ferrous ion. The ferrous ion formed in this way can readily be oxidized to give the ferric hydroxide. The more pronounced effect of stirring upon the hydrolysis is undoubtedly due to the formation of large particulate masses by collisions of smaller iron hydroxide particles, and to more rapid oxidation of any ferrous ion formed.

As pointed out by Harvey (1945), iron ascorbate has less tendency to become hydrolyzed in saline media, if the solution is freshly prepared. On standing, stock solutions of iron ascorbate, originally tinted but a very light green, assume deep brown colorations. It was found that ferric ascorbate was not decomposed for periods up to one week, but after longer periods the formation of centrifugable iron hydroxide followed the course of iron citrate solutions. Iodoform tests on both the

[2] The radioactive iron metal used in this investigation was supplied by the Oak Ridge National Laboratory on allocation from the Isotopes Division, U. S. Atomic Energy Commission.

TABLE I

The hydrolysis of iron complexes in sea water solutions

Complex of ferric ion	Time elapsed since addition of iron complex (days)	% of original iron remaining in supernate	Remarks
Citrate	0	100	Light, stirred
	1	64	
	2	29	
	3	20	
	4	11	
	6	6	
Citrate	4	50	Dark, unstirred
	4	10	Light, stirred
	4	31	Light, unstirred
Ethylene diamine tetra-acetate	0	100	Light, stirred
	1	88	
	3	80	
	6	15	

ferric citrate and ferric ascorbate solutions were positive within two weeks after preparation.

Ethylenediamine tetra-acetic acid chelates with trivalent iron and this complex was studied as a potential iron source. However, after six days 85 per cent of the iron had hydrolyzed at a pH of 8.25. These results are consistent with the experiments of Jacobson (1951) who found instability of the complex in basic aqueous solutions.

It should be noted that in all cases involving the complexing of iron, the iron and complexing agent were mixed in a 1:2 molar proportion. The iron concentration was 2.2 micromoles/liter.

UPTAKE OF IRON BY ASTERIONELLA JAPONICA

In order to establish a minimal cell content of iron necessary for further growth, varying amounts of iron in the form of iron citrate were added to the culture bottles with an initial diatom inoculum of 300–400 cells/ml. The culture medium was assayed for iron colorimetrically before the addition of iron citrate. If the nutrient water showed no iron content, it was used in the growth experiments. It was normally found that inshore surface sea water filtered through a Whatman No. 42 paper was free of iron within the limit of the analytical method (0.1 micromole per liter).

The results of a typical experiment are given in Table II. No growth was observed until the initial iron concentration exceeded 0.8 micromole per liter. Both growth and final population of the cells could be controlled by the initial iron concentration. The iron per diatom, when leveling-off of the exponential growth occurred, averaged 1.0×10^{-7} micromoles per cell. We have assumed that essentially all the iron was removed by the biomass. Since the maximum cell densities were not reached until 6 or 8 days after inoculation, the greater part of the iron available to the diatoms was in particulate form.

TABLE II

Iron uptake by Asterionella japonica

Culture bottle	1	2	3	4	5	6
Initial iron in micromoles/liter	0.8	1.6	2.2	2.5	3.4	3.4
Final diatom population cells/ml.	0	14,700	21,700	18,200	41,900	41,100
Final iron content of diatoms micromoles/cell $\times 10^7$	0	1.1	1.0	1.4	0.81	0.82

Initial diatom inoculum: 300 cells/ml.
Initial phosphate: 2.62 micromoles/liter.

Humic acid has been reported as a complexing agent which makes iron more readily available to various plants and micro-organisms. Harvey (1937b) found increased growth with an artificial iron humate, but the effect was less than that of the iron citrate complex. Although the chemical formulae for the various iron complexes that are present in both natural and synthetic preparations are undetermined, it was desirable to ascertain whether iron humate might be a suitable nutrient source for *Asterionella japonica*. The synthetic humic acid and iron humate solutions were prepared according to the method of Burk, Lineweaver and Horner (1932). The iron content of the iron humate solutions was determined colorimetrically after a wet digestion with 3:1 nitric: perchloric acid mixture. The iron humate does not give centrifugable products from sea water solutions when prepared in this fashion.

The results of the humate experiments are given in Table III. Iron citrate was added to control cultures. The initial phosphate level was 2.7 micromoles per liter and the diatom inoculum was 750 cells/ml. Humate as the source of iron did not promote growth. However, the excess humic acid constituents did not deter diatom growth, as is seen in the results of bottle 5. We may therefore conclude that this form of iron humate is not available for metabolic utilization by *Asterionella japonica*.

Freshly prepared radioactive iron ascorbate was added to the culture bottles to give an initial concentration of 1.45 micromoles per liter. Three moles of ascorbate were present for each mole of iron. Exponential diatom growth was observed up to 30,000 cells per ml.; however, the cells appeared misshapen and unhealthy. No detectable iron was removed from the solution by radioactive assay of

TABLE III

Diatom growth experiments with ferric humate as the source of iron

Culture flask	1	2	3	4	5	6
Iron form	Humate	Citrate	Humate	Citrate	Citrate + Humic acid	Humate
Initial iron concentration micromoles/liter	12.5	14	12.5	14	14	6.3
Final concentration diatoms cells/ml.	0	81,000	0	62,000	70,000	0

the supernatant solution. To confirm this finding the diatoms were filtered, digested in the 3:1 nitric: perchloric acid mixture and the iron activity was determined radiometrically. The cell content of the accumulated radio-iron averaged 1.9×10^{-9} micromoles per cell. This is about 2% of the minimal iron content as determined from the uptake experiments previously described. The initial amount of particulate iron in the culture flasks was 1.8 micromoles per liter added as ferric chloride solution.

One final experiment was undertaken to confirm the observation that in the absence of complexed ferric iron and in the presence of particulate iron, diatom growth could proceed. Cells from a growing sub-culture were placed in a dialyzing sac which held 50 ml. of nutrient solution such that there was an initial diatom population of 800 cells/ml. The sac was held in place by a rubber stopper in a 250 ml. volumetric cylinder which contained 210 ml. of solution. Freshly prepared iron citrate was introduced into either the dialyzing sac or the cylinder to give an iron concentration of 2.2 micromoles per liter. In the former case the ionic complexed iron penetrated the membrane to give a concentration of 0.01 micromole per liter after 2 days, *i.e.*, 20% of the iron diffused into the outer cylinder. Here normal exponential growth occurred, whereas when the diatoms could receive only the complexed iron as in the latter case, no population increases were noted.

Discussion

From the above experiments it is evident that the organic iron complexes used were not available as a growth nutrient to marine diatoms, whereas particulate and/or colloidal forms of iron are utilized in their metabolism.

Cooper (1935) found that the ratios of iron to phosphorus, in plankton samples consisting almost entirely of diatoms, were 4.2 and 4.4 in two separate determinations. From the experiments of phosphate uptake by *Asterionella japonica*, 5×10^{-8} micromoles of phosphate per cell were necessary for division. Combining this with the minimum iron content of 10×10^{-8} micromoles/cell, we calculate for our cultural populations

$$\mathrm{Fe/P} = \frac{55.85 \times 10 \times 10^{-8}}{30.98 \times 5 \times 10^{-8}} = 3.6$$

The striking agreement between these values suggests that this method of study of cultural diatom populations represents a fair simulation of ocean growth.

Cooper (1948) attributes the extreme variability in iron content in the upper layers of marine waters as due to the random distribution of particles of a large size (*i.e.*, 160 μ), as large as some sand grains. It would be of interest to know what size of iron hydroxide particle might be necessary for diatom division. We may assume a density of the particle as 1.1 and an iron content of 50 per cent. Since 1.0×10^{-7} micromoles or 55.85×10^{-13} grams are needed per cell we have, where r is the radius of the particle,

$$55.85 \times 10^{-13} = \tfrac{4}{3}\pi r^3 \times 1.1 \times \tfrac{1}{2} \quad \text{or} \quad r = 2 \times 10^{-4}\ \text{cm.} = 2\mu.$$

From the above relationships it is noted that a particle of 4 μ radius would furnish enough iron for three divisions.

The use of iron content as a parameter in a nutrient index of a productivity equation now presents itself. However, unlike necessary ionic species, the absolute concentration of iron does not reflect its availability. A direct study is needed of the number of particulate iron particles and their size distribution. One possible approach to this problem has already been initiated in this laboratory. Sea water is filtered through a cellulose membrane filter which retains particles of 0.1 μ in diameter. Following the filtration the membrane is washed with distilled water and dried. The membrane is then cleared with polyoxyethylene sorbitan monolaurate which is saturated with potassium thiocynate and is acidified with 3 drops HCl per 100 ml. The typical red iron thiocyanate color is produced about any iron particle. By the use of this technique, a study of the size distribution of the iron particles in the euphotic zone of Pacific coastal waters will be made.

Summary

1. With the use of radioactive iron it was found that the marine diatom *Asterionella japonica* utilized only particulate and/or colloidal iron as a growth nutrient, whereas ionically complexed ferric ion as the citrate, ascorbate, or artificial humate was not available for uptake.

2. A minimal content of iron per cell needed for further division was established as 10×10^{-8} micromoles per liter. The ratio of the minimal iron to the minimal phosphate agrees substantially with the iron-to-phosphate ratio in natural plankton samples.

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PHARMACOLOGY OF HAEMAL VESSELS OF STICHOPUS CALIFORNICUS

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Myogenic hearts are, in general, inhibited by acetylcholine whereas neurogenic hearts are accelerated by it (Prosser, 1942). Similarly, myogenic hearts are less sensitive to ether than are neurogenic hearts (Needham, 1950). No vessels of echinoderms have previously been studied in respect to contraction mechanisms or pacemakers. The haemal vessels (lacunar system) of certain holothurians contain a fluid which is rich in protein and hemoglobin-containing corpuscles. In *Stichopus californicus,* a species often 18 inches long when extended, two large vessels serve the intestine; these are the internal vessel and the larger external vessel which is connected by many small branches to the intestine and in its lower portion also to the respiratory tree. The internal vessel appears not to be spontaneously contractile but the external vessel and its branches to the intestine contract rhythmically. The contractions are very weakly peristaltic but appear to be predominantly local, like segmentation in a vertebrate intestine. Circulation in the ramifying haemal system has not been studied in detail but the rate of flow of fluid must be very slow.

Methods

Contractions of the external vessel were observed with a dissecting microscope and contractions indicated on a kymograph by a signal magnet. Some observations were made *in situ,* some with the vessel attached to pieces of the intestine, but most with isolated pieces of the vessel, approximately 5 cm. in length, mounted in a wax chamber through which sea water and appropriate solutions of drugs in sea water could pass at a constant rate of flow.

Rates of beat were obtained from records of a minimum of 5 contractions after equilibrium was attained with each application of a drug. Isolated preparations varied in their activity, but many continued to beat actively for several hours.

Results

The frequency of beats in the external haemal vessel of Stichopus is low, ranging from 4 to 5.5 beats per minute at 18° to 20° C.

Acetylcholine strongly inhibits the beat of the haemal vessel, reversible inhibition of 16 to 36 per cent in rate occurring in three preparations at a dilution in sea water of 10^{-12}. At 10^{-14} two of four preparations showed 25 per cent inhibition. A dilution of 10^{-16} was without effect as ascertained with four preparations. At higher concentrations (10^{-10} to 10^{-6}), the beats became irregular, amplitude was markedly reduced and in the higher range the heart stopped in a relaxed state. The magni-

tude of the effect in the concentration range 10^{-10} to 10^{-6} varied considerably and it is not likely that the Stichopus vessel, despite its high sensitivity, would make a satisfactory assay material for acetylcholine.

Physostigmine in moderate concentrations inhibited the vessel, but a concentration of 10^{-10} in sea water had no effect alone; however, after treatment with this concentration of eserine, acetylcholine at 10^{-10}, which had previously been ineffective, caused a 25 per cent slowing.

Nicotine inhibits the Stichopus vessel. Thresholds varied, but in two preparations, significant inhibition was observed at a dilution of 10^{-11}. One preparation gave the following percentages of inhibition at the dilutions indicated: 10^{-9}, 15 per cent; 10^{-8}, 43 per cent; 10^{-7}, 57 per cent; 10^{-6}, total cessation of beat.

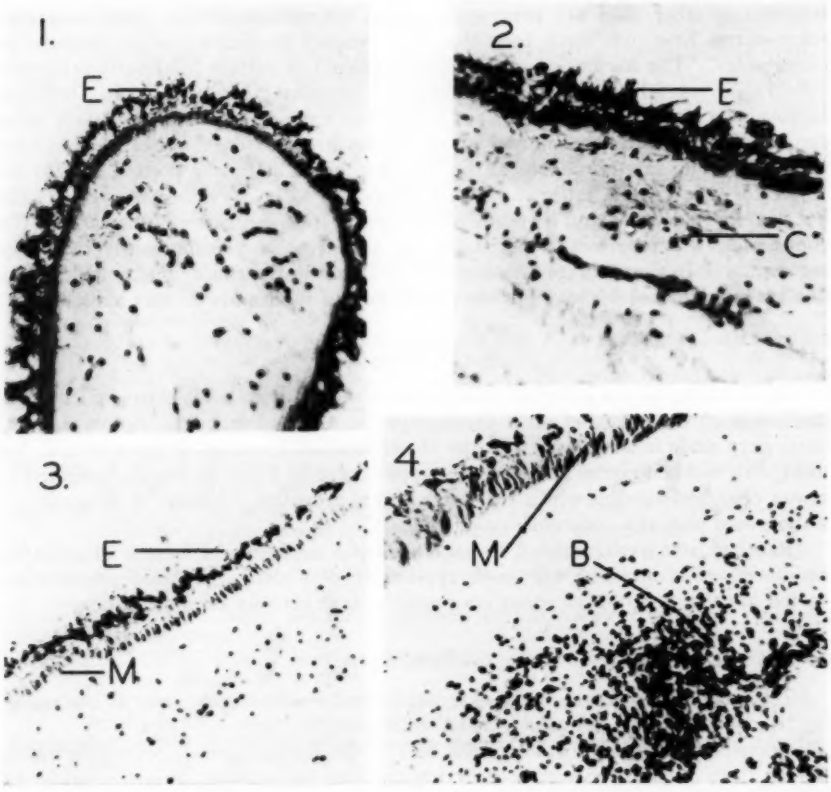

PLATE I. Sections of vessels of *Stichopus californicus*. × 80. E—epithelium; M—circular muscle; C—connective tissue; B—region of formation of blood cells.

FIGURE 1. Cross section of small branch vessel.

FIGURE 2. Longitudinal section close to edge of wall of large vessel.

FIGURE 3. Longitudinal section through center of vessel.

FIGURE 4. Longitudinal section in region of hemopoiesis.

Tetramethyl ammonium bromide likewise inhibited the Stichopus vessel at concentrations of 10^{-8} and higher.

Tetraethyl ammonium bromide antagonizes the nicotinic action of acetylcholine on the heart of Venus and in mammalian sympathetic ganglia (Welsh and Taub, 1950). On the Stichopus vessel tetraethyl ammonium in concentrations which might have been effective against acetylcholine proved toxic. Dilutions of 10^{-4} stopped the vessel, 10^{-5} slowed it and 10^{-6} failed to cause recovery from acetlycholine inhibition.

The electrocardiogram of myogenic hearts usually consists of slow waves, while that of neurogenic hearts is often oscillatory (Prosser, 1950). Electrocardiograms from the haemal vessels of Stichopus were recorded with a Sanborn electrocardiograph. The vessels were mounted in mineral oil and contact made by wicks soaked in sea water. Results were extremely variable and inconstant but in several favorable preparations single slow waves of negativity were correlated with contractions. In none was oscillatory activity observed.

Histological examination of the external haemal vessel showed an outer epithelial layer, the cells of which have small protruding processes (Figs. 1, 3). These projections give a villous appearance to the epithelium and greatly increase the surface exposed to the body cavity. Beneath the epithelium is a single layer of circular muscle fibers but no longitudinal muscle fibers (Figs. 1, 2, 3); this observation explains the segmental nature of the contractions. Most of the vessel is composed of large, loosely-packed connective tissue cells which contain regions of blood-cell manufacture (Fig. 4). The lumen is lined with a thin unicellular layer of endothelium. It cannot be said positively that nerve cells are absent from the vessel, but certainly no aggregations of them occur.

Conclusions

The external haemal vessel of the holothurian, *Stichopus californicus,* beats spontaneously. It consists of villous epithelium, circular muscle, and connective tissue with regions of hemopoiesis. The rhythmic contractions are inhibited by acetylcholine with a threshold concentration of the order of 10^{-14}. Physostigmine potentiates the response to acetylcholine while both nicotine and tetramethyl ammonium chloride inhibit the beat of the vessel. The action potential appears to be a simple wave of negativity. By analogy with the pharmacology of other hearts it is tentatively concluded that the Stichopus haemal vessel is myogenic.

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CYTOLOGICAL STUDIES ON MUCUS FORMATION AND SECRETION IN BUSYCON [1]

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The formation and secretion of mucus, sometimes of great importance to physiologists and ecologists, are poorly understood. A few studies have been made of mucous glands and of their activity in various organisms. Thus, Re (1951) has studied secretion of mucus in the amphibian oviduct, and Patten (1950) has described some aspects of histogenesis in mucous epithelia of a urodele and two rodents. Vaubel (1933) observed the behavior of mammalian synovial cells in tissue culture. Studies on invertebrates have included observations on some cytological aspects of the secretion *in vivo* by an oligochaete (Millott, 1948). The mucus-producing ability of mollusks is well known; the pallial mucous cells of a pelecypod, *Anodonta*, have recently been studied by Defretin and Riff (1948). The prosobranch (gastropod) hypobranchial gland contains masses of large mucus-producing cells, and for this reason deserves special attention (Ronkin and Ronkin, 1951). Thus Tarao (1935) described the hypobranchial gland of the abalone, *Haliotis japonica*, and attempted to analyze the time-sequence of the secretion using Hirsch's (1931) methods for the classification and analysis of secretory activity. An earlier morphological description of the gastropod hypobranchial gland (Dakin, 1912) includes some reference to previous work. The present study is largely cytochemical in nature and is directed mainly toward an understanding of the chemical processes leading to formation of mucus in the hypobranchial gland of *Busycon*. Since this gland has not been adequately described, it is necessary to include a brief morphological survey; this adds to our meager knowledge of molluscan histology.

Materials and Methods

Obtaining the excised gland. *Busycon canaliculatum* (Linnaeus), a large marine snail, is easy to handle and was readily available during most of the year by dredging near Woods Hole, Mass. To obtain the fresh gland the animal was held on a board, with the columella vertical and apex pointed downward. The outer whorls of shell were cracked with a hammer; then a *dulled* axe blade was inserted into the external groove separating adjacent whorls, and twisted. After one or two whorls of shell were removed in this manner the animal was grasped at the operculum, its columellar muscle worked loose with the fingers, and the intact snail then "unscrewed" from the remaining shell. One could now make out the pallial organs

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[2] With the technical assistance of Elizabeth P. Ronkin.

(rectum, hypobranchial gland, ctenidium and osphradium, from the midline to the animal's left), through the translucent roof of the pallial cavity. The portion of the mantle bearing the hypobranchial gland was excised for study.

Preparation of material for microscopic study. The excised gland was easily maintained alive in sea water at room temperature (20 to 25° C.) for five days, during which time ciliary activity and secretion of mucus were evident. Attempts to obtain separate, living mucous cells and to observe them with the compound microscope failed, probably because of the fragility of these cells. For the same reason, attempts to obtain fresh-frozen sections with the freezing microtome also failed. However, it was possible to obtain frozen sections (at 25 μ) of glandular epithelium which had previously been fixed in 10% neutral formalin for 8 to 24 hours. Some attempts to stain the living cells with dilute solutions of neutral red, Janus green B, and toluidine blue O were also made, but the dye failed in each case to penetrate the mucous layer surrounding the cells. For most histological and cytological studies the gland was placed in a fixative solution immediately after excision.

Gilson's mercuric-nitric solution gave good fixation most often when compared with other fixatives (acetone at 5° C., alcohol-formalin, Formol-Zenker, *i*-pentane at − 170° C., lead acetate-formalin, 5% mercuric chloride, osmic-sublimate,[3] propylene glycol at − 20° C., Regaud's, Susa's, and toluene at − 20° C.). Ethanol was used for dehydration, followed by clearing in toluene and infiltration and imbedding in paraffin. Sections were cut at 7 to 10 μ.

For general studies of histological and cytological features, sections were stained with toluidine blue O (0.1% aqueous) followed by potassium ferrocyanide (1% aqueous), Weigert's hematoxylin, and metanil yellow (0.25% in 0.25% acetic acid), with the usual intermediate rinses and subsequent dehydration, clearing, and mounting. Mayer's mucicarmine was occasionally used as a substitute for the toluidine blue O-potassium ferrocyanide combination, and was satisfactory for many purposes. Mallory's triple stain was used for histological features.

In addition to these, and to certain special methods described in the literature, the following methods are referred to in this paper:

a. Alcian blue 8GS.[4] This stain, whose use for mucus has recently been suggested by Steedman (1950), was employed in 1% aqueous solution; metanil yellow was used as a counterstain.

b. Amylase digestion. Hydrated sections were incubated in human saliva at 37.5° C. for one hour, then for an additional half-hour in distilled water to remove the salivary mucus.

c. Bauer-Feulgen stain. This procedure for demonstrating glycogen and mucus follows the schedule outlined by Glick (1949), using 4% chromic acid.

d. Hyaluronidase digestion. Hydrated sections were incubated in 0.01% bovine testicular hyaluronidase[5] at 37.5° C. for the desired time. They were then rinsed several times in distilled water before further treatment.

[3] Ludford-Mann-Kopsch method (Bensley and Bensley, 1938).

[4] A new dye derived from monastral fast blue B (Haddock, 1948). Samples were kindly furnished by Dr. H. A. Lubs of E. I. du Pont de Nemours and Co., Inc., and by Dr. H. F. Steedman of the University of Glasgow. It has recently become commercially available as a biological stain in the United States.

[5] Worthington Biochemical Sales Co., Freehold, N. J.

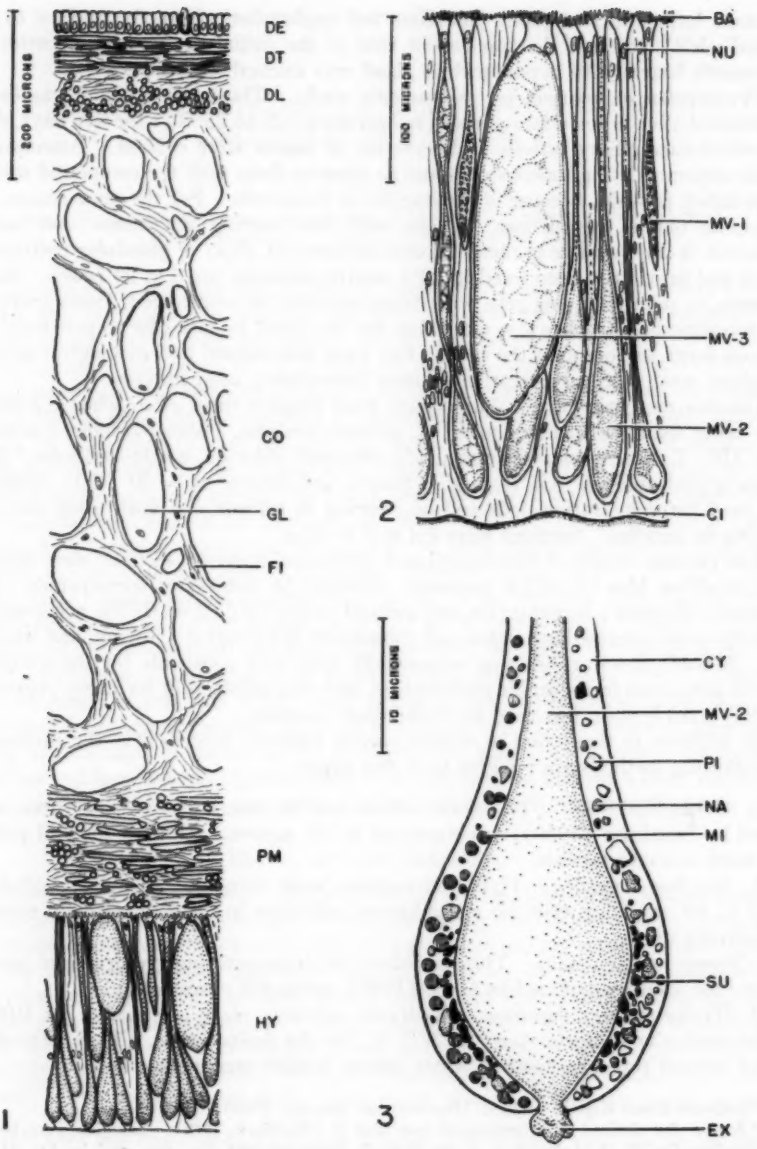

FIGURE 1. Semi-diagrammatic transverse section through a portion of the mantle of *Busycon* bearing the hypobranchial gland. CO, connective-tissue layer; DE, dorsal epithelium; DL, dorsal longitudinal muscular layer; DT, dorsal transverse muscular layer; FI, fibrous tissue; GL, glycogen cell; HY, hypobranchial gland; PM, pallial muscular layer.

e. Nadi test. This procedure for demonstrating cytochrome oxidase, using *a*-naphthol and *p*-amino dimethylaniline, is described by Glick (1949). Tissues were fixed in 10% formalin and sectioned with the freezing microtome, stained, and mounted in Apáthy's gum-syrup (Lillie, 1948).

f. Prussian-blue test for intracellular iron. Hydrated sections were immersed first in ammonium sulfide, then in potassium ferrocyanide solution (0.75% in 0.25% HCl), and finally counterstained in eosin Y, with intermediate rinses in distilled water.

When available, Commission-certified stains were used. Unless otherwise stated, stained sections were dehydrated in a series of ethanols, cleared in xylene and mounted in Clarite. When enzymatic digestion was used, comparisons were made with undigested sections from the same block.

Observations

General structure of the hypobranchial gland (Fig. 1). The glandular and ciliated epithelium which faces the pallial cavity consists of a single layer of long, slender cells, arranged normal to the free epithelial surface, and is bounded dorsally by a basement membrane. Above the basement membrane is a relatively thin sheet of fibrous and muscular tissue, with fibers arranged parallel to the free surface. The thickest layer of the mantle comes next and consists of a loose network of fibrous connective tissue. Most of the spaces bounded by the fibers are filled by cells which contain glycogen in large amounts. Next dorsally is a muscular and connective-tissue layer. Finally there is a very thin layer of glandular epithelial cells which bounds the dorsal (outer) surface of the mantle.

Examination of the inner epithelial layer (*i.e.*, the hypobranchial gland itself; Fig. 2) revealed that it is composed of ciliated cells, several types of mucous cells, and some rather interesting cellular fragments. The ciliated cells are conical. The bases of the cones pave the surface of the gland and their apices are prolonged as slender stalks whose extremities probably attach to the basement membrane. The nucleus of each ciliated cell is to be found about half the distance from the surface to the basement membrane.

Types of mucous cells. The following working classification of mucous cells is proposed:

Mucous cells of type 1 frequently extend only part way from the basement layer to the free surface of the gland. The mucous vacuole is slender and usually spindle- or club-shaped; its contents appear gray and foamy in unstained material, dark red with Mayer's mucicarmine, and frequently blue (*i.e.*, without metachromasy) with toluidine blue O.

Figure 2. Cell types in the hypobranchial gland. BA, basement membrane; CI, ciliated cells; MV–1, 2, 3, mucous vacuoles of cells of types 1, 2, and 3, respectively; NU, nucleus of a type-2 cell. Note the masses of nuclei about two-thirds the distance from the basement membrane to the ciliated surface.

Figure 3. Semi-diagrammatic drawing of the distal end of a type-2 cell. CY, cytoplasm; EX, extruded mucus; MI, mitochondrion (mitochondria are cross-hatched and are omitted from the right half of the drawing); MV–2, mucous vacuole; NA, nadi-positive granule (stippled); PI, pigment granule (omitted from the left half of the drawing); SU, sudanophilic granule (solid black). Golgi material is not represented.

Mucous cells of type 2 generally extend the full depth, or nearly the full depth, of the glandular layer. The large mucous vacuole is club-shaped, and usually can be followed from its distal enlargement almost to the base of the cell; although one or two broad regions may be seen, usually the vacuole tapers rapidly and terminates in isolated granules close to the nucleus. In some cells the nucleus is at some distance from the basement membrane; in such cells all the mucus is distal to the nucleus, and the cell is connected to the basement layer by a slender stalk. The mucus stains metachromatically (reddish violet) with toluidine blue O and bright red with mucicarmine; it appears gray and foamy in unstained material. One may suggest that cells of type 1 include the youngest in a series of mucous cells of different ages, and that type-2 cells are older; evidence in support of this relationship will be offered elsewhere in this paper.

TABLE I

Staining and other reactions of mucous-vacuolar contents in the various kinds of glandular cells described

Stain and condition	Type 1	Type 2	Type 3
a. Mayer's mucicarmine	Reddish violet	Red	Like type 2
b. Alcian blue 8GS	Blue-green, but parts of some vacuoles unstained	Blue-green	Like type 2
c. Toluidine blue O	Some blue and nongranular; some blue with fine or coarse metachromatic granules; the latter having decreasing amounts of blue-staining interstitial material; some with metachromatic, foamy contents with optically empty interstices	Reddish violet (metachromatic)	Like type 2
d. Toluidine blue O, preceded by 24 hours hyaluronidase digestion	Metachromasy in granules, but paler; blue color faded or absent	Pale metachromasy	Like type 2
e. Bauer-Feulgen	Red granules, of uniform size within each cell	Red and finely granular	Clumps of fine, pink granules
f. Bauer-Feulgen, preceded by 24 hours hyaluronidase digestion	No visible change on exposure to enzyme	Pale pink	Optically empty

Mucous cells of type 3 have very large, ellipsoidal or irregular vacuoles whose contents, when present, appear to have the same staining properties as those of type 2, but seem to be less dense or more open in texture. They are found close to the basement layer but may extend most of the distance to the surface. Whether cells of type 3 are part of the same series as those represented by types 1 and 2 cannot be stated for lack of evidence. However, the similarity between types 2 and 3 with respect to staining reactions and susceptibility to hyaluronidase (Table I) suggests chemical similarities in the mucus.

Usually the mucous cells are normal to the free surface, but some portions of the gland (especially those at its left, or ctenidial, edge) are thrown into folds which bear a superficial resemblance to the gastric crypts in vertebrates. In such locations the mucous cells usually appear to be of type 2, but they are much shorter than

elsewhere in the gland and seem to pour their secretions into the crypt rather than directly into the pallial cavity.

Masses of nuclei, paler and more elongated than those found at the bases of mucous cells, were frequently seen occupying clusters of cytoplasmic strands, at positions about halfway from the basement membrane to the free surface. Many nuclei were also encountered in the secreted mucus lying on the surface of the gland. Some of these nuclei belong to ciliated cells, but none could be traced to mucous cells. It is possible that the remaining nuclei and cytoplasmic strands are remnants of former mucous cells whose distal portions have become emptied or lost.

The contents of the mucous vacuoles. In formalin-fixed material, frozen-sectioned and unstained, the contents of the mucous vacuole are gray, refringent, and appear foamy in texture. In toluidine blue-, alcian blue-, or mucicarmine-stained cells the appearance is slightly distorted, probably owing to the preceding dehydration, and the contents tend to look fibrous. It is the fibrous phase, corresponding to the continuous or suspending phase of the "foam," which becomes stained with uniform intensity throughout the vacuole. It is thus unlikely that the suspended spheres in the mucous vacuole, which remain unstained, are of mucous nature. The mucus in the three types of mucous cells stains differently with the various staining methods used, as indicated in Table I.

Cytoplasmic inclusions other than mucus (Fig. 3). The fresh gland appears yellowish brown to the unaided eye. Microscopic examination of portions of the gland fixed in 10% formalin and sectioned with the freezing microtome revealed that the scanty cytoplasm of the stained or unstained mucous cells of all types contains many canary-yellow granules. The yellow color which they confer upon the cytoplasm is visible under low power. These granules vary widely in size, the largest being about 5 μ in diameter and the smallest at the limit of microscopic resolution in white light. The larger granules appeared to be concentrated about the distal, more bulbous portion of each type-2 cell. The following description refers to type-2 cells, which are those most commonly found.

Mitochondria were demonstrable with Regaud's hematoxylin. Spherical basophilic granules, 0.4 to 2.1 μ in diameter, were concentrated and more easily distinguishable in the distal half of the mucous cell than in the proximal, or basal, half. They were not as easily demonstrated with Altmann's aniline acid fuchsin.

The possible presence of Golgi material was shown by osmiophilia in the mucous cells of deeper portions of the "crypts" at the left edge of the hypobranchial gland. The densely packed, intensely brownish-black granules occupied the distal portion of the cytoplasm of each cell, outside the mucous vacuole. Since these sections had been bleached with potassium permanganate subsequent to osmic-acid treatment, the distribution of osmiophilic granules may indicate the location of Golgi material (Lillie, 1948).

Formalin-fixed, frozen-sectioned glandular material stained with Sudan black B (Wislocki and Dempsey, 1948) revealed blackish-green granules distributed throughout the cytoplasm, but concentrated especially in a broad belt encircling the distal portion, but not the tip of the mucous vacuole. Most of the sudanophilic granules were 0.4 to 1.3 μ in diameter, with a few as large as 2.5 μ. Several belts of granules measured were 4 to 12 μ wide, compared with mucous-vacuolar diameters

of about 17 μ in this portion of the cell. Sudanophilia is presumptive evidence of lipid nature.

Similarly fixed and sectioned material was treated with the "nadi" reagent for the determination of cytochrome oxidase. Treated sections showed intense, blue granules, usually varying in diameter from 1.7 μ downward, throughout the cytoplasm. Near the expanded distal portion of the cell, granules up to 3.5 μ in diameter were found.

Iron was not present in any of the mucous cells in amounts large enough to be detected by the Prussian-blue test. Prussian-blue granules were present, however, in the ciliated epithelium of the adjacent ctenidium, portions of which were sometimes included on the same slides with hypobranchial-gland material.

Attempts to demonstrate glycogen in the mucous cells with the Bauer-Feulgen test were unsuccessful. The presence of Bauer-Feulgen-positive material in the underlying connective tissue, and its absence in control sections which had been subjected to digestion with salivary amylase, suggested that glycogen was absent from the mucous cells.

Observations of secretion. A few cells were observed in which the secretion of mucus had been stopped by histological fixation. The freshly extruded mucus usually appeared as a small wisp protruding from the center of the distal tip of the cell, but occasionally as a small rounded cap or button covering the tip of the cell. There was no evidence of a large amount of mucus being extruded from a cell in a short time, suggesting that the production of mucus is a slow and continuous type of activity, probably varying in rate from time to time, and with secretion occurring only occasionally. Millott (1948) has seen sphincter-like structures surrounding the mouths of mucous cells in *Lumbricus;* no analogs of these structures could be seen in material studied here. It is possible that the ciliated cells, by the lateral exertion of pressure, may aid in mucus secretion in *Busycon* in the way Millott has suggested for the oligochaete, but no evidence for this mechanism was observed.

Discussion

If one defines a mucous cell for the purposes of this study as one whose secretion-vacuolar contents stain metachromatically with toluidine blue O, then it is probable that all kinds of secreting cells observed were mucous in nature. In *Haliotis,* however, Tarao (1935) found several kinds of non-mucous, secreting cells which he was able to classify according to the types of secretion granules. The non-mucous cells were intermixed with mucous, ciliated, and sustentacular cells in the hypobranchial gland. In *Busycon* all the observed glandular cells appear to be associated with mucus production, and no sustentacular cells could be distinguished, though these may have been represented by some of the massed nuclei referred to above.

The probable involvement of the nucleus in mucus formation, suggested by Tarao, is supported by the location of the nucleus near the slender base of the mucous vacuole in *Busycon.* Painter (1945) has suggested that formation of secretory materials is related to nuclear metabolism in Drosophila and in the honeybee. Wilson (1928) has referred to the earlier literature on nuclear function in relation to secretory activity in protozoa.

The genetic relationships of the different kinds of glandular cells in *Busycon* do not seem as clear as those described in *Haliotis* by Tarao. The observations re-

ported here suggest that mucous cells of types 1 and 2 are earlier and later stages in the maturation of cells of a single type. The position of cells of type 3 is uncertain; they may constitute a separate category, or they may represent an older stage than type 2, in the same series. The evidence for the relation between cells of types 1 and 2 lies in (1) the generally greater length of type-2 cells, compared with type 1; (2) the failure to observe extrusion of secretion from type-1 cells; (3) the heterogeneity of vacuolar contents of type 1, with respect to its staining reactions with alcian blue and toluidine blue, suggesting a transitional state.

The last observation confirms those of Vaubel (1933), who found that young synovial cells in tissue culture contained granules orthochromatic to toluidine blue, whereas older cells contained metachromatic granules. In *Busycon* the acquisition of metachromasy by the mucous-vacuolar contents, which is apparently in progress in type-1 cells, reflects a chemical change in the mucous precursor. The property of metachromasy is correlated in general with the binding of sulfate in polysaccharides (Lison, 1933; see also Michaelis and Granick, 1945, and Masamune *et al.*, 1947), and it seems likely that sulfate binding may be an important step in the formation of mucus in progress in cells of type 1.

The reported presence of glycogen in the mucous cells of *Anodonta* (Defretin and Riff, 1948) indicates a difference between *Busycon* and this pelecypod material. The failure to detect glycogen in the hypobranchial mucous cells and its presence in the overlying tissues suggest that the mucous cells may obtain their carbohydrates by diffusion from the neighboring cells. The presence of galactose in other molluscan tissues (Bell and Baldwin, 1941; Masamune *et al.*, 1947) may mean that galactogen is of general importance in this group of animals, in addition to glycogen; galactokinase and galactose-isomerase systems might well be sought.

Finally, the peculiar distribution of lipid material in relation to the mucous vacuole in type-2 cells suggests that lipid metabolism may be involved in the formation or secretion of mucus, or both. Although the granules which appeared in these cytochemical tests may well be crystalline precipitates and may not exist in the living cell, their locations suggest the distribution of constituents present in life. The distribution of mitochondria and that suggested for cytochrome oxidase may indicate the loci of biochemical systems involving aerobic syntheses. Studies on lipid transformations and respiratory metabolism in these cells and further studies of the spatial distribution of the participating substances may clarify the mechanisms of the formation and secretion of mucus.

Summary

1. The hypobranchial (mucous) gland of *Busycon canaliculatum* (L.) is located in the pallial cavity. It contains ciliated cells and three types of mucous cells.

2. Mucous cells of type 1 are thought to be precursors of type 2 because of the relative sizes and positions of the two types of cells, and because mucous vacuoles of type-1 cells appear to have varying degrees of resemblance to those of type-2 cells. Evidence concerning the relationships of type-3 cells is lacking.

3. The commonest type of mucous cell (type 2) is long and slender with a bulbous tip, one or two swellings along its length, and a basal nucleus. Most of the cell is occupied by a mucous vacuole, whose contents stain metachromatically (reddish violet) with toluidine blue O, red with mucicarmine, blue-green with alcian blue 8GS, and are partly digested by hyaluronidase. The surrounding cytoplasm con-

tains yellow pigment granules, mitochondria, Golgi apparatus, and nadi-positive granules. The distribution of sudanophilic material is peculiar in that a belt of lipid granules surrounds the sub-distal portion of the bulbous tip of the vacuole. No glycogen could be demonstrated in these cells.

4. It is possible that the activities of mucous cells require diffusion of carbohydrate materials from adjacent cells. The results of the cytochemical tests suggest that aerobic utilization of carbohydrates (possibly galactose) or lipid materials, and the binding of sulfate groups may be of importance in the formation of mucus from its precursors.

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NEUROSECRETION. XI. THE EFFECTS OF NERVE SECTION ON THE INTERCEREBRALIS–CARDIACUM–ALLATUM SYSTEM OF THE INSECT LEUCOPHAEA MADERAE [1]

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In the course of a histological study of the intercerebralis-cardiacum-allatum system in specimens of the insect *Leucophaea maderae* in which the nervi corporis cardiaci had been sectioned (B. Scharrer, 1946a), it became evident that this system offers a favorable material for the investigation of a question of general importance, namely that of the transport of neurosecretory material along nerve fibers.

The intercerebralis-cardiacum-allatum system of insects is in many ways analogous to the hypothalamo-hypophyseal system of vertebrates (Hanström, 1941; M. Thomsen, 1943; Scharrer and Scharrer, 1944; Cazal, 1948). In both cases histological observations suggest that the stainable material produced by the neurosecretory cells of the pars intercerebralis and the hypothalamus, respectively, passes along the axons of these cells into organs in which it is stored. In the insects these axons form the nervi corporis cardiaci (nervi corporis cardiaci I, Hanström) and reach not only the corpora cardiaca but in part also the corpora allata. The corpora cardiaca act in the capacity of storage organs for neurosecretory material, in at least a number of species (B. Scharrer, 1951; 1952c); in the vertebrates the neurohypophysis fulfills a corresponding function (Bargmann and Scharrer, 1951).

If the neurosecretory substance actually "migrates" along the axons of the cells from which it originates, it might accumulate when its flow is blocked, as for instance by section of the nerve fibers. Hild (1951), working with frogs, and Stutinsky (1951), using frogs and rats, were indeed able to demonstrate the accumulation of neurosecretory material in the pituitary stalk proximal to the site of section. Scharrer and Wittenstein (1952) reported corresponding observations in dogs in which the pituitary stalk had been severed. However, when compared with insects, vertebrates do not offer equally favorable conditions for the study of this problem. In the operation on vertebrates not only nerve fibers, but also blood vessels must, by necessity, be severed. It is difficult to assess the part played by the interruption of the vascular channels in causing histological changes in addition to those following the severance of the nerve fibers. In the intercerebralis-cardiacum-allatum system of insects the effect of the interruption of the fiber tracts can be studied without reference to changes that might be caused by interference with the blood supply. A further advantage is that the intercerebralis-cardiacum-allatum system is paired in many insects, including Leucophaea. Consequently, the neurosecretory pathway can be sectioned on one side without damage to the other side which serves as control in the same animal. The effects of the interruption of this neurosecretory

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pathway were studied, therefore, in Leucophaea with regard to (a) the transport of the stainable material produced by neurosecretory cells along the axons of these cells, (b) the storage function of the corpora cardiaca, and (c) the reaction of the corpora allata.

Material and Methods

Nerve sections were performed in 124 nymphs and in 115 adults of Leucophaea of both sexes. The nervi corporis cardiaci which enter the corpora cardiaca soon after emerging from the postero-ventral surface of the brain are not easily accessible. The most suitable approach was found to be through a window cut anteriorly in the head capsule; the brain had to be somewhat tilted so that the nerves could be severed. As a rule the operation was performed in nymphs at the beginning of an intermolt period and in adults shortly after emergence. The operated specimens were allowed to survive from 5 days to 10 months. The heads were fixed in Zenker-formol or Bouin, and serial sections of 5 micra were stained with Gomori's (1941) chrome alum-hematoxylin-phloxin method or with Foot's (1933) modification of Masson's trichrome stain. While the Gomori technique is not more specific for the identification of neurosecretory material than the Masson method, it affords a more precise characterization of this substance in both vertebrates and invertebrates than the Masson and other previously used methods and is, therefore, preferable. The sections on which the present study is based include cases with unilateral and with bilateral interruption of the nervi corporis cardiaci.

Since the amount of neurosecretory material normally present in the intercerebralis-cardiacum complex varies to some degree with age, physiological conditions, etc., the cases with unilateral nerve section are particularly instructive for the determination of the histological changes resulting from the interruption of the neurosecretory pathway.

Observations

The effects of the severance of the nervi corporis cardiaci on the histological appearance of the intercerebralis-cardiacum-allatum system of Leucophaea are twofold. One result of this operation consists in marked effects on the distribution of the neurosecretory material, the other concerns a structural change in the corpora allata. Male and female animals respond to the operation in the same manner.

The topography of the organs in question as well as the levels at which sections for photomicrographs (Figs. 2–9) were selected are indicated diagrammatically in Figure 1. The paired nervi corporis cardiaci which originate from the neurosecretory cells of the pars intercerebralis cross before leaving the brain. Their axons enter the elongated corpora cardiaca where they can be traced on each side for a considerable distance as a well defined nerve bundle. In all probability some of the fibers reach the corpora allata, but since in Leucophaea the corpora allata are directly attached to the posterior end of the corpora cardiaca, there is no distinct nervus corporis allati.

As has been pointed out previously (Scharrer and Scharrer, 1944) the distinctive feature of the nervous pathway connecting the pars intercerebralis of the brain with the corpora cardiaca-allata is the presence of varying amounts of neurosecretory material throughout its course. Within the corpus cardiacum this material, which stains deep blue with the Gomori technique and bright red with the Masson stain,

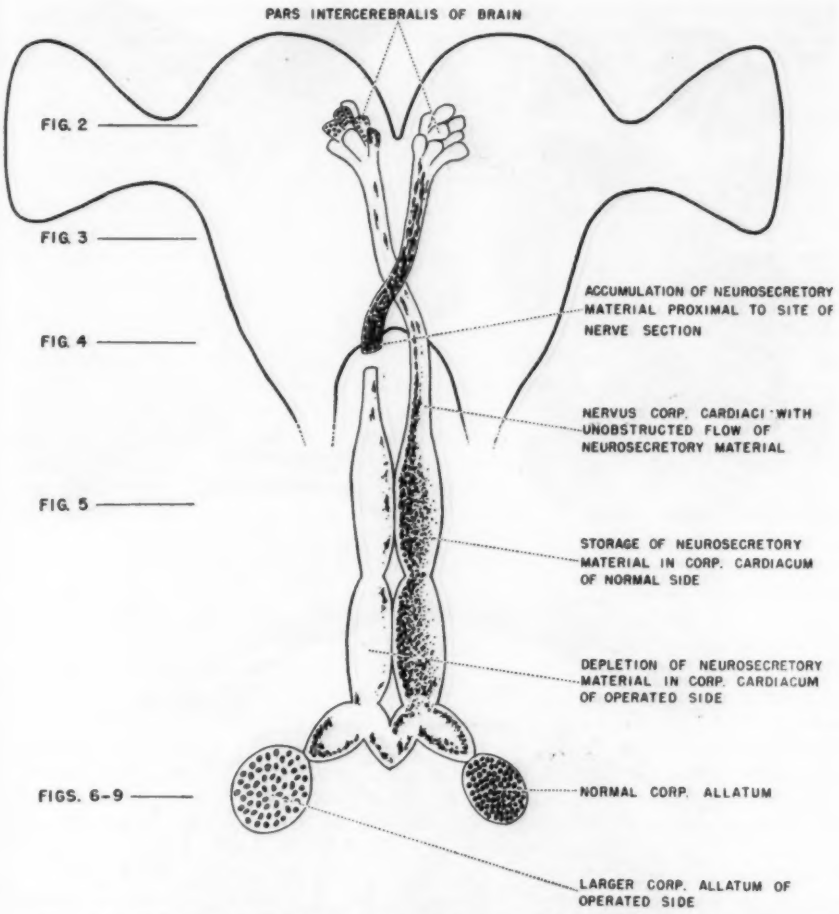
PARS INTERCEREBRALIS OF BRAIN
FIG. 2
FIG. 3
FIG. 4
FIG. 5
FIGS. 6-9
ACCUMULATION OF NEUROSECRETORY MATERIAL PROXIMAL TO SITE OF NERVE SECTION
NERVUS CORP. CARDIACI WITH UNOBSTRUCTED FLOW OF NEUROSECRETORY MATERIAL
STORAGE OF NEUROSECRETORY MATERIAL IN CORP. CARDIACUM OF NORMAL SIDE
DEPLETION OF NEUROSECRETORY MATERIAL IN CORP. CARDIACUM OF OPERATED SIDE
NORMAL CORP. ALLATUM
LARGER CORP. ALLATUM OF OPERATED SIDE

FIGURE 1. Diagram of the dorsal aspect of the intercerebralis-cardiacum-allatum system of *Leucophaea maderae*. On the left side the nervus corporis cardiaci is severed with the result that neurosecretory material is increased proximal to and depleted distal to the site of nerve section. On the operated side the corpus cardiacum is decreased, the corpus allatum increased in size. Levels of the photomicrographs shown in Figures 2–9 are indicated; the sections illustrated in these figures were made from paraffin-embedded material, cut at 5 micra, and stained with the chrome alum-hematoxylin-phloxin method of Gomori.

follows in its distribution the course of the fiber bundles of the nervi corporis cardiaci. This topographic relationship is particularly evident in the anterior portion of the corpora cardiaca. Masses of neurosecretory material are usually confined to this nerve bundle for some distance and, farther distally, "fan out" into the cardiacum tissue. Neurosecretory material is, as a rule, more abundant in the marginal zones,

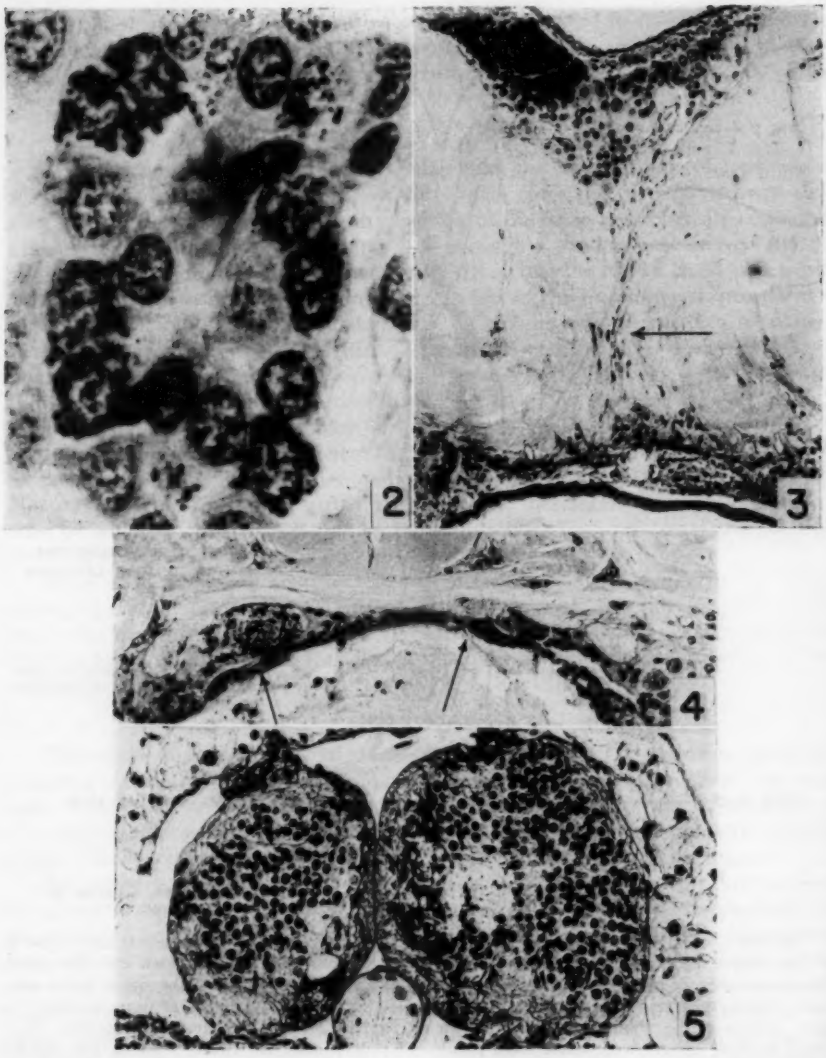

FIGURE 2. Neurosecretory cells of the pars intercerebralis of Leucophaea on the unoperated side with conspicuous cytoplasmic inclusions. The operated side of this case shows very little neurosecretion. Adult female, fixed in Bouin 5½ months after operation. Photomicrograph, × 1000.

FIGURE 3. Brain of adult female fixed in Bouin five months after operation. Level of decussation of nervi corporis cardiaci. At the arrow the tract of the operated side, which contains an increased amount of dark staining neurosecretory material, crosses the almost empty tract of the intact side (compare with Figure 1). Photomicrograph, × 100.

particularly those facing the lumen of the dorsal vessel which is surrounded by the corpora cardiaca. In the same sections the corpora allata are free of neurosecretory substance.

I. *The effect of the severance of the nervi corporis cardiaci on the distribution of neurosecretory material*

If the neurosecretory pathway is interrupted in the manner described, the result is an accumulation of neurosecretory material proximal to the site of operation and a marked depletion of this substance in the distal portions of this system (B. Scharrer, 1952a). These histological changes suggest that the normally occurring flow of neurosecretory material along the axons of the cells in which it originates must have been blocked by the nerve section.

The accumulation of neurosecretory material at the proximal end of the cut fiber tract is particularly noticed in cases where the interval between the operation and the fixation of the tissues is short, *i.e.*, several days. Figure 4 shows a cross section through the nervi corporis cardiaci near the ventral surface of the brain a short distance from their exit. On the side where the operation was performed the nerve contains considerable masses of neurosecretory material, while the nerve on the unoperated side contains only a small amount. In cases where the interval between the operation and the fixation of the organs is several months, the accumulated neurosecretory substance usually extends to a more proximal point with the result that the severed tract, throughout its course within the brain, shows an increased amount of inclusions as compared with the control side (Fig. 3). This phenomenon of accumulation of the substance is also observed in cases of bilateral nerve section which serve to corroborate the results of unilateral operations. In specimens which had been allowed to survive for the longest period of time in this series of experiments (up to 10 months) the accumulation effect may be absent. It is to be assumed that the neurosecretory cells whose axons have been cut eventually cease to produce, or produce a smaller amount of the material which is normally transported to the corpora cardiaca. This assumption is supported by the observation that in some of the long interval cases studied the cells of the pars intercerebralis contain more neurosecretory material on the normal than on the operated side (Fig. 2).

While the section of the nervi corporis cardiaci results in an increase of neurosecretory material in the proximal part of the system, the portions distal to the level of transection show a reduction in the amount of this substance. Figure 5 demonstrates the difference between normal and operated side in that the corpus cardiacum whose nerve has been severed contains practically no material stainable with the

FIGURE 4. Cross section of nervi corporis cardiaci (arrows) in postero-ventral part of brain near site of their emergence. The severed tract on the left side, shown proximal to the level of operation, contains considerably more dark staining material than the intact tract on the right side. Female fixed in Zenker-formol five days after nerve severance. Photomicrograph, × 160.

FIGURE 5. Cross section of corpora cardiaca of female with unilateral severance of nervus corporis cardiaci, operated five months before fixation in Bouin. The corpus cardiacum on the operated (left) side is smaller and contains very little neurosecretory material in contrast to the normal control gland on the right side which shows dark masses of this substance in the medial zone. Photomicrograph, × 210.

FIGURES 6–9. Corpora allata of Leucophaea with unilateral severance of the nervus corporis cardiaci. The organs on the operated side at left are "swollen" with more cytoplasm and looser arrangement of nuclei than those on the normal right side. Figure 6: Female fixed in Zenker-formol five days after nerve severance. Photomicrograph, × 100. Figure 7: Female fixed in Bouin five months after nerve severance. Photomicrograph, × 100. Figures 8 and 9: Male fixed in Bouin six months after operation. The contrast between "severed" (Fig. 8) and normal (Fig. 9) corpus allatum is particularly marked. Photomicrograph, × 375.

Gomori technique. It also shows that the corpus cardiacum on the operated side is smaller than on the normal side. This effect of nerve severance on the corpus cardiacum is different from that observed on the corpus allatum (see below) and may be the result of prolonged inactivity as a storage center. The posterior part of the corpora cardiaca of specimens with unilateral operation may contain neurosecretory substance on both sides, but since the organs are continuous at the posterior end the material on the operated side probably is derived from the intact side.

II. *The effect of the severance of the nervi corporis cardiaci on the structure of the corpora allata*

In histological preparations, the corpora allata of normal specimens of Leucophaea are, as a rule, conspicuous by the density of their nuclei which make the stained organs look dark. After severance of the nervi corporis cardiaci the histological appearance of the corpora allata changes markedly. The organs appear larger and lighter in color. This effect is brought about by an increase in cytoplasm relative to the number of nuclei per unit of area. The nuclei are especially loosely arranged in the periphery of these organs. It seems that the number of nuclei per gland remains the same after nerve severance, and that the increase in organ size constitutes an increase in cytoplasm, which may be due merely to uptake of water or may indicate an actual production of cytoplasmic material. The nuclei also differ from normal ones after nerve severance; in the operated organs they tend to be more vesicular and their nucleoli are more distinct.

The difference between a normal corpus allatum and one whose connection with the brain has been interrupted can be particularly well demonstrated in cases of unilateral nerve severance. The illustrations (Figs. 6 to 9) show organs from animals which had been allowed to survive the operation for five days, five months, and six months respectively. The structural change is already evident a short time after nerve severance; it is especially marked in the case with an interval of six months after the operation.

The size difference between these normal and "severed" corpora allata was roughly estimated in the following manner. In 5 micra serial sections of the organs, camera lucida outline drawings of every fourth section were made. In the case of an imperfect section the one preceding or following it was substituted. The areas in square millimeters were determined planimetrically and the values were added up for each organ measured. The figures for three representative cases were as follows:

		Normal	Operated
Case 737	♀ (int. 5 ds.)	5,954.83 sq. mm.	10,335.46 sq. mm.
Case 424	♀ (int. 5 ms.)	6,148.37 sq. mm.	8,148.37 sq. mm.
Case 465	♂ (int. 6 ms.)	1,122.58 sq. mm.	5,729.02 sq. mm.

The differences between the values for the normal and the operated side indicate a substantial increase in the size of those corpora allata whose nervous connection with the brain had been severed.

Discussion

The observation that the distribution of neurosecretory material in the intercerebralis-cardiacum complex of Leucophaea changes in the manner described after

the nervi corporis cardiaci are severed, clearly indicates that normally a transport of this material takes place along the axons of the neurosecretory neurons. This conclusion is further supported by the corresponding data in vertebrates as stated in the introduction. In frogs (Hild, 1951), in rats (Stutinsky, 1951) and in dogs (Scharrer and Wittenstein, 1952) the accumulation of neurosecretory material proximal to the site of severance of the pituitary stalk is evident soon (several days) after the operation and disappears after a longer interval. The same is true in Leucophaea. A depletion of this material in the portion distal to the transection, such as that observed in Leucophaea, has so far not been clearly demonstrated in vertebrates, but attention should be called to the fact that the observation of such quantitative differences is more difficult in animals where the organ system in question is unpaired and consequently no comparison can be made with a control organ in the same animal.

As far as other insects are concerned, a comparable transport of neurosecretory material from the brain to the corpus cardiacum must be assumed to take place in a variety of species where, as in Leucophaea, the occurrence of this material in the nervi corporis cardiaci can be demonstrated (Cazal, 1948; Stutinsky, 1952).

The concept of the transport and storage of neurosecretory material in the corpus cardiacum makes understandable the physiological result observed in Leucophaea after the interruption of the nervi corporis cardiaci (B. Scharrer, 1946a). These experiments gave no indication of an acute hormone deficiency and no deficiency should be expected since the operation does not result in the immediate depletion of neurosecretory material in the components of the system. The extent to which the evaluation of the corpus cardiacum as a reservoir for hormones originating in the brain can explain other known physiological observations has been discussed in some detail elsewhere (B. Scharrer, 1952b, 1952c).

The interpretation of the hypothalamo-hypophyseal system of vertebrates (Bargmann and Scharrer, 1951) and of the intercerebralis-cardiacum-allatum system of insects (B. Scharrer, 1951; 1952b; see also Scharrer and Scharrer, 1944) in this new light has led to a re-examination of a comparable situation in the eyestalk of crustaceans. In this group of arthropods the sinus gland has long been considered as the source of a number of hormones, while the functional role of the X organ remained obscure ever since its discovery by Hanström (1931). Recent investigations show that hormones known to be present in the sinus gland, especially the molt-inhibiting hormone, are only stored there; they actually originate in the neurosecretory cells of the X organ from which they are transported to their reservoir via the sinus gland nerve (Passano, 1951a, 1951b; Bliss, 1951; Travis, 1951; Frost, Saloum and Kleinholz, 1951).

Morphological evidence of the presence of neurosecretory material in this nerve has been obtained in a number of brachyurans (Enami, 1951; Bliss, 1951; Passano, 1951a). Nerve section in crustaceans (Passano, 1951a) seems to have an effect on the distribution of the neurosecretory material comparable to that in insects as reported here. The information available now is sufficient proof for the existence of a third neurosecretory system (X organ-sinus gland system) which corresponds in many details to the neurosecretory systems in insects and in vertebrates.

As to the effect of nerve section on the corpora allata, one may inquire into the nature of the structural changes observed in these organs after their connection

with the brain has been severed. Do they represent a specific reaction attributable to the absence of nervous or of endocrine (neurosecretory) stimulation and, if this is so, does the histological response to this operation reflect a change in the physiological activity of the corpora allata?

The reaction to the severance of the nervi corporis cardiaci cannot be explained as an unspecific effect, possibly brought about by the surgical procedure, because if this were true unilateral transection would have to affect both corpora allata in a similar way. Whether the interruption of the nerves in itself or the disturbance in the flow of neurosecretory substance along these nerves causes the change, cannot be decided at this time. There is information, however, regarding the physiological activity of "severed" corpora allata. It has been previously reported (B. Scharrer, 1946a) that the separation from the brain does not render the corpora allata of Leucophaea functionally incompetent. Bilateral section of the nervi corporis cardiaci neither prevents egg maturation nor nymphal molting, both being processes governed by corpus allatum hormone. This result is not unexpected in view of the fact that allatum implants can substitute for the intact gland in allatectomized animals (B. Scharrer, 1946b, 1946c). In these cases the isolated corpora allata react like intact glands, *i.e.*, they furnish hormone which reaches the circulation in sufficient amounts to be effective.

A physiological difference between normal and "severed" organs which may concern hormone release rather than hormone production can be observed in last instar nymphs. It is generally assumed that, in the course of insect development, metamorphosis takes place because the corpora allata in the last larval stage no longer release an adequate amount of juvenile hormone. The manner in which this presumed restraining influence on the corpora allata is mediated has not been determined. The effect of the cutting of the nervi corporis cardiaci shows, however, that this regulatory mechanism requires the intactness of the nervous pathway. Last instars of Leucophaea in which the corpora allata are severed from the brain may undergo supernumerary molts and become "nymphoids" instead of adults (B. Scharrer, 1946a), an indication that the severed corpora allata can release a significant amount of juvenile hormone at a stage when they normally would not do so. This result can be interpreted in several ways. Under normal conditions the brain may regulate the release of corpus allatum hormone by means of the nervi corporis cardiaci. In view of the peculiar nature of this pathway the action on the corpora allata can be either nervous or neuro-endocrine in character. Another possibility is that the potency of the severed corpus allatum is increased to such a degree that the juvenile hormone released during the last stage reaches the threshold necessary for an additional nymphal molt, while normally this level is not reached.

In the latter case the histological changes following nerve severance would have to be interpreted as a sign of increased glandular activity rather than of deficiency. This has been done by some authors who observed structural differences similar to the ones described here in Leucophaea, when they compared the corpora allata of specimens in inactive and active phases of their development (Wigglesworth, 1934; Mendes, 1948), of males and females (Pflugfelder, 1938, 1948; Schrader, 1938; Hanström, 1942; Palm, 1948) or of normal and castrate specimens (Pfeiffer, 1940; E. Thomsen, 1942; Day, 1943; see also Vogt, 1942) of certain insect species.

An effect of nerve severance on the structure of the corpus allatum has been

reported only in one other instance. Day (1943) observed an increase in the size of the corpus allatum comparable to the one in Leucophaea, when the "recurrent" nerve of the fly Sarcophaga was cut. In the adult flies this nerve contains both nervi corporis cardiaci at the level of the operation. Therefore, Day's experiment constitutes a denervation of the corpus allatum, while the severance of the nervi corporis cardiaci in Leucophaea does not deprive the corpora allata of fibers they may receive from the stomatogastric system. Whether or not this anatomical difference, determining different degrees of denervation, accounts for the differences in the physiological effects observed in Leucophaea on the one hand and in Sarcophaga on the other is not certain. While the stimulation of the ovaries of Leucophaea by the corpora allata continues after section of the nervi corporis cardiaci this is not the case in Sarcophaga where, according to Day, nerve severance has the same effect as extirpation of the corpus cardiacum-allatum. With respect to Leucophaea one may assume that the physiological effect which parallels the morphological change of the corpora allata following nerve severance constitutes an increase rather than a decrease in glandular activity.

SUMMARY

1. The interpretation of the intercerebralis-cardiacum-allatum system of insects as a functionally related group of neuroglandular organs, in which the corpus cardiacum serves as a reservoir for neurosecretory material, is supported by the results of severance of the nervi corporis cardiaci. The insect, *Leucophaea maderae*, in which the components of this organ system are paired is favorable for the study of these relationships since one side can be left intact and compared with the operated side.

2. Following the interruption of the neurosecretory pathway at the level where the nervus corporis cardiaci enters the corpus cardiacum, the distribution of the neurosecretory material changes markedly in comparison with the normal situation. Proximal to the site of nerve section there is an accumulation, and distal to it there is a drastic depletion of neurosecretory material. This result offers conclusive evidence that in the intact system the neurosecretory substance originating in cells of the pars intercerebralis of the brain is transported via their axons (forming the nervi corporis cardiaci) to the corpora cardiaca where it is stored. This interpretation of the morphological situation makes it understandable that no physiological indication of hormone deficiency was observed as a consequence of the nerve transection (B. Scharrer, 1946a).

3. Severance of the nervi corporis cardiaci also results in histological changes in the corpora allata. The organs become larger and seem to contain more cytoplasm than normal glands. Their nuclei are more vesicular and the nucleoli more distinct. These changes do not indicate an organ deficiency, since "severed" corpora allata of Leucophaea are functionally equal to, or perhaps even more competent than, normally innervated glands.

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THE EFFECT OF SALTS AND ORGANIC SOLUTES ON THE MIGRATION TIME OF THE SLIME MOLD DICTYOSTELIUM DISCOIDEUM [1]

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As Raper (1935) showed when he first described the amoeboid slime mold *Dictyostelium discoideum*, this particular species is unique in that it possesses a migration stage in its life cycle. The independent, separate amoebae of the vegetative stage stream together to form cell masses or pseudoplasmodia, and these pseudoplasmodia migrate over the surface of the substratum until the final culmination stage. In culmination there is both a rising into the air and a cell differentiation; the result is a fruiting body in which a delicate stalk made up of large vacuolate amoebae supports an apical mass of encapsulated amoebae or spores.

The duration of this migration period has always been known to be extremely variable, from a matter of a few hours to approximately two days, and Raper (1940) demonstrated that low temperature and high humidity favored prolonged migration to some extent. While these observations have been confirmed we have also found that the concentration of solutes in the medium proves to be a far more important limiting factor, and by varying this environmental condition it has been possible to control and to greatly extend the duration of migration.

Methods

The organisms were cultivated on standard medium (Bonner, 1947)[2] with *Escherichia coli* at room temperature for two days, at which time the amoebae had usually begun to aggregate. Plugs of the standard agar covered with the aggregating amoebae were cut out with a glass ring 10 mm. in diameter and placed on a circular coverslip of equal diameter, which was then set on the medium to be tested. The coverslip was used so that no diffusion could take place between the plug of standard medium and the test medium. All the test plates were incubated at 17° C. The technique thus far is essentially similar to that used by Bonner *et al.* (1950).

After 24 hours a sufficient number of migrating pseudoplasmodia had moved on to the test medium so that the plug and coverslip could be removed. Each of the migrating pseudoplasmodia that remained was marked by placing a drop of ink on the bottom of the petri dish at 24-hour intervals until fruiting occurred. In cases where the pseudoplasmodia split longitudinally all the parts were followed.

[1] The preliminary experiments of this study were carried out at Princeton University with the help of a grant from the American Cancer Society.

[2] There is an error in the formula of the culture medium in Bonner (1947) and KH_2PO_4 should be substituted for the incorrect K_2HPO_4.

The test media were prepared by adding the different solute concentrations to 2% Difco Bacto-Agar and each solute was tested separately. All solutes were chemically pure.

Results

First a test was made using the standard medium. As can be seen from the table, in 137 cases the mean migration time was 2.0 days (± a standard deviation of 1.3) and the range extended from 1 to 5 days. This is in keeping with the previous reports in the literature, but if the migrating pseudoplasmodia were allowed to migrate in 2% agar alone, the mean time was extended to 10.3 days (see table) and in one instance the migration lasted 20 days. As a matter of fact the mean value is in this instance of dubious significance because 29% of the pseudoplasmodia disappeared completely, presumably from a combination of loss of cells by straggling in the posterior track and the loss of protoplasm used in the energy of locomotion. The smaller the original size of the pseudoplasmodium, the sooner it would disappear, but there was no evident correlation between duration of migration before fruiting, and the size of the pseudoplasmodium.

As can be seen from the table and from Figure 1, if any solute was added to the 2% agar, the greater the concentration of the solute the shorter the mean duration of migration. This was true for the three organic non-electrolytes tried (dextrose, sucrose, dl-alanine) and the three electrolytes (NaCl, Na_2HPO_4, $CaCl_2$). None of the non-electrolytes was significantly different from any other, but as a group the salts were from 4 to 6 times more effective at a given concentration. Among the salts, NaCl and $CaCl_2$ were very similar in their effectiveness, while Na_2HPO_4 was twice as effective.

If concentrations of solutes higher than those given in the table were used, only a few pseudoplasmodia crawled off the standard medium plug even though they readily left plugs from the same standard plate when put on media of lower concentrations. There was one exception to this: in the case of the 6% dextrose a good number of pseudoplasmodia migrated off the plug, but as soon as they touched the surrounding medium they fruited. This happened in 78 cases without any deviations and represents a condition of minimum migration. It was also noticed on repeated occasions that prolonged migration never occurred over the surface of glass, but that if the pseudoplasmodium reached the side of the petri dish, it fruited shortly thereafter.

Discussion

The cessation of migration and the onset of fruiting is a major step in the differentiation process of *Dictyostelium*. It is of interest therefore to observe that the concentration of the solutes can play a part in the initiation of differentiation. Considering the importance of concentration, the effect would appear to be an osmotic phenomenon, but this would neither account for the more effective nature of the salts, nor the fact that the salts differ among themselves when their effective osmotic pressure is calculated. In the future it is hoped that by testing other substances it will be possible to understand more thoroughly the mechanism of initiation of fruiting and the basis for the variability of effectiveness of different substances.

There is a parallel to the phenomena found here in the work of Smith and Grenan (1949), who have found that the concentration of the medium will affect

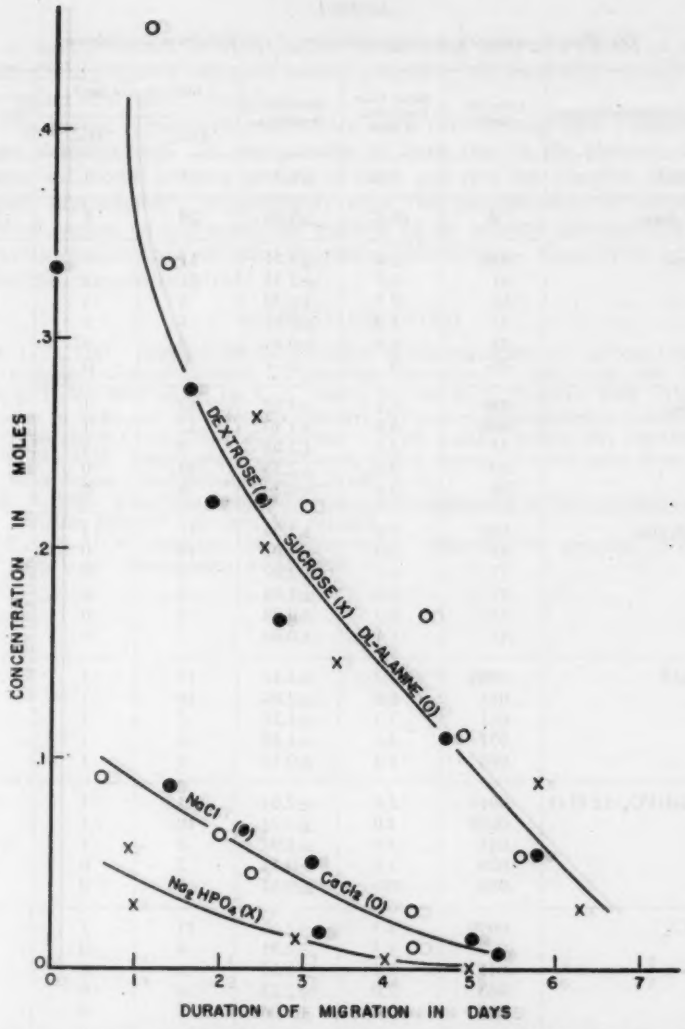

FIGURE 1. A graph showing the relationship between the concentration of solute (moles) in a 2% agar test medium and the duration of migration of the slime mold *Dictyostelium discoideum* in days.

the degree of spreading of the plasmodium in the true Myxomycete, *Physarum polycephalum*. Again with increasing concentrations there is a decrease in spreading, which suggests the possibility that in *Dictyostelium* the solutes affect the motility of the cell mass and therefore indirectly initiate final differentiation.

TABLE I

The effect of solutes on the migration time of Dictyostelium discoideum

% Weight of solute in 2% agar	Conc. in moles	Mean time migration in days	Standard deviation	Migration in days		No. of cases
				Max.	Min.	
Std. medium		2.0	±1.3	5	1	137
2% Agar alone	0	10.3	±5.40	20	1	142
1% dextrose	.056	5.8	±2.71	11	1	79
2%	.11	4.7	±2.33	11	1	53
3%	.17	2.7	±1.30	8	1	154
4%	.22	1.9	±0.92	4	1	56
5%	.28	1.6	±0.54	3	1	53
6%	.33	0	0	0	0	78
1% sucrose	.029	6.3	±3.02	13	1	120
3%	.088	5.8	±2.40	12	1	59
5%	.15	3.4	±2.05	9	1	58
7%	.20	2.5	±2.28	10	0	29
9%	.26	2.4	±1.70	6	0	33
0.5% dl-alanine	.056	5.6	±2.34	11	1	82
1%	.11	4.9	±2.02	10	0	94
1.5%	.17	4.4	±2.07	8	0	64
2%	.22	3.0	±1.74	7	0	59
3%	.34	1.3	±0.99	3	0	43
4%	.45	1.1	±0.80	3	0	17
0.05% NaCl	.0086	5.3	±2.41	11	1	73
0.08%	.014	5.0	±2.04	10	1	76
0.1%	.017	3.2	±1.76	7	1	90
0.3%	.051	3.1	±1.28	6	1	71
0.5%	.086	1.4	±0.75	4	1	74
0.05% $Na_2HPO_4 \cdot 12\ H_2O$	.0014	5.0	±2.64	11	1	109
0.1%	.0028	4.0	±2.24	10	1	49
0.5%	.014	2.9	±1.92	8	1	141
1%	.028	1.0	±0.42	2	0	113
2%	.056	0.9	±0.54	2	0	37
0.1% $CaCl_2$	.0090	4.3	±2.60	11	1	63
0.3%	.027	4.3	±2.01	8	0	63
0.5%	.045	2.4	±1.53	6	0	47
0.7%	.063	2.0	±1.15	4	0	26
1.0%	.090	0.6	±0.06	2	0	24

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Summary

1. In the life history of *Dictyostelium discoideum* there is a period of migration in which the aggregated cell mass wanders about in the form of a sausage prior to the formation of a final fruiting body.

2. The period of migration was known to be variable, and now a limiting factor has been demonstrated. It was possible to show that in the absence of solutes migration will occur for long periods of time, and that the duration of migration decreased approximately exponentially with the concentration of added solute. The effect cannot be accounted for entirely as an osmotic phenomenon because different substances have different degrees of effectiveness, electrolytes being more effective than non-electrolytes.

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THE EFFECT OF PARROT FISH THYROID EXTRACT ON THE RESPIRATORY METABOLISM OF THE WHITE RAT [1, 2]

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The discovery of a discrete, encapsulated thyroid gland in the parrot fish (Matthews, 1948) made possible studies on the teleost thyroid which had hitherto been undertaken only with the greatest difficulty. Extracts of the fish thyroid became obtainable for the first time with relative ease. Such extracts, when injected into white grunts, significantly elevated the oxygen consumption in a certain proportion of the fish so treated (Smith and Matthews, 1948). The thyroids of parrot fish were found to concentrate iodine in a manner reminiscent of similar behavior in the glands of mammals (Matthews and Smith, 1948). There was thus established a certain parallelism between the action of teleost and mammalian thyroids and it was thought worthwhile to investigate the effect of parrot fish thyroid extract upon the respiratory metabolism of the white rat.

Methods

The extracts used in these studies were prepared from thyroid glands collected in Bermuda and Bimini. With rare exceptions, the glands were taken from *Pseudoscarus guacamaia*. As previously described (Smith and Matthews, 1950), the thyroid glands were removed immediately after the death of the fish and were placed in either acetone or absolute alcohol. Dehydration and defatting continued for at least two days with alternate changes of acetone or absolute alcohol. At the end of this period the glands were dried in partial vacuum and stored, either powdered or whole, in a desiccator until prepared for injection. The final average weight of the glandular material was 17 per cent of the wet weight of the gland when removed from the fish.

Extracts were prepared by dissolving the desired amount of dried glandular tissue in 2 cc. of 2N NaOH and bringing the resultant solution to neutrality with concentrated HCl. This procedure usually precipitated most of the dissolved material, but the resultant suspension was fine enough to be drawn through a 20 gauge needle and injected into the test animal. As a rule approximately 100 mgm. of dried gland were prepared in this manner. The final volume of solution injected amounted to about 2.0 cc. This solution was injected intra-abdominally into adult male white rats, bred from laboratory stock. The rats stood the injection well and showed no sign of distress immediately afterward or at any later time. The animals were kept in individual cages under laboratory conditions during the time necessary to complete the experiment. During this period they were given the usual food and water *ad libitum*.

[1] With the support of a grant from the American Philosophical Society.
[2] Contribution No. 173, Bermuda Biological Station for Research.

Mammalian thyroid extracts were prepared in the same manner, the necessary amount of commercial desiccated mammalian thyroid (tablet form) being dissolved in NaOH and neutralized with HCl. The synthetic thyroxine used in these experiments was prepared by Roche-Organon, one cc. of the solution containing 2 mgm. of thyroxine. Material for control injections was prepared by adding to the required amount of NaOH sufficient concentrated HCl to bring the pH of the solution to about 7.0.

Oxygen consumption was determined by placing the rat in a desiccator (volume 9500 cc.) through which air was forced under a slight positive pressure sufficient to give a flow of 500 to 670 cc. per minute. The flow was adjusted so that CO_2 concentration at equilibrium usually fell between 0.7 and 1.3 per cent. The animals quickly became accustomed to the apparatus and after briefly exploring their surroundings settled down and usually spent most of the time sleeping throughout the remainder of the test. Samples of air were taken after the rats had been in the chamber for two hours. They were analyzed with a Haldane-Henderson gas analyzer to determine the amount of oxygen which was removed and the amount of carbon dioxide which was added to the air during its passage through the chamber, the rate of flow at the time of sampling having been previously determined. It was then possible to calculate both oxygen consumption and CO_2 production during the test. The respiratory quotient and the oxygen concentration per hour per gram of body weight were then determined. The validity of the method is predicated, of course, on the assumption that no sudden change in oxygen consumption occurred during the test period, particularly within 10 minutes before collection of the sample.

The tests were run in the morning, after the rats had been left overnight in their cages with food and water. They were not fasted, therefore, prior to the test, since it is well known that rats under laboratory conditions usually feed at night and spend the daylight hours in sleep. Since repeated tests were desired on successive days extending over a period of a week or more, depriving the rats of food for this period would have produced such abnormal behavior as to invalidate the results. The relative constancy of the oxygen consumption of each rat over a period of several days prior to any experimental procedure indicated that the metabolic conditions prevailing during the tests were reasonably similar.

Results

The purpose of the experiments was to determine the effect of parrot fish thyroid extract on the oxygen consumption, respiratory quotient and weight of adult male white rats and to make a comparison with the effects produced by synthetic thyroxine and desiccated mammalian thyroid powder.

Table I gives the results of 86 determinations of oxygen consumption and R.Q. in 22 non-treated rats. Their weights ranged from 250 to 500 grams (average 300 grams). Average oxygen consumption was 0.98 cc./gm./hr. and the average R.Q. was 0.84. As may be seen from an inspection of Table II, the values for the various test series before injection of thyroid preparations were in every instance close to these figures.

In Table II are shown the results of injecting various substances, known or suspected to contain active thyroid hormone, as compared to the effect of injecting

TABLE I

Oxygen consumption and respiratory quotient in non-treated white rats

Number of determinations	86
O_2 consumption (cc./gm./hr.) Mean ± SE_M	0.98 ± 0.0129
Respiratory quotient Mean ± SE_M	0.84 ± 0.0070

TABLE II

Oxygen consumption and respiratory quotient in treated rats

Rat no.	Material injected	Before injection		Days after injection											
				1		2		3		4		7		10	
		R. Q.	O_2 cons. cc./hr./gm.	R. Q.	O_2 cons. cc./hr./gm.	R. Q.	O_2 cons. cc./hr./gm.	R. Q.	O_2 cons. cc./hr./gm.	R. Q.	O_2 cons. cc./hr./gm.	R. Q.	O_2 cons. cc./hr./gm.	R. Q.	O_2 cons. cc./hr./gm.
8	Neut.	0.85	0.93	0.85	0.97	0.84	0.97								
4	NaOH	0.84	0.91	0.82	0.97	0.85	1.02								
1	2 cc.	0.82	1.08	0.77	0.93	0.81	1.03	0.84	0.93	0.85	0.80	0.74	0.92		
2A		0.83	1.12			0.76	0.99	0.79	0.91	0.79	0.95	0.84	0.89		
4A		0.78	1.12	0.81	1.01	0.79	1.08			0.81	1.12	0.84	1.01		
6A		0.84	0.98	0.76	1.01	0.81	0.89	0.80	0.91	0.81	0.95	0.79	0.96		
Ave.		0.83	1.02	0.80	0.98	0.81	1.00	0.81	0.92	0.82	0.96	0.80	0.95		
2	Thyroxine	0.76	1.02	0.79	1.34	0.69	1.44	0.72	1.16					0.93	0.99
7	1 mgm.	0.80	1.14	0.74	1.28	0.68	1.36	0.75	1.34					0.86	0.93
11	1 mgm.	0.85	0.91	0.79	1.22	0.69	1.39	0.74	1.15					0.81	0.90
0	0.2 mgm.	0.78	0.95	0.76	1.21	0.74	1.24	0.80	1.00						
13	0.2 mgm.	0.85	0.95	0.73	1.18	0.73	1.06	0.76	1.00						
Ave.		0.81	0.99	0.76	1.25	0.71	1.30	0.75	1.13					0.87	0.94
7A	Mammalian	0.83	0.90	0.69	1.13	0.74	1.08	0.72	0.97			0.94	1.03	0.95	0.88
8A	Thyroid 130 mgm.	0.84	1.06	0.80	1.31	0.82	1.25	0.75	1.36	0.78	1.28	0.76	1.45		
Ave.		0.84	0.98	0.75	1.22	0.78	1.16	0.74	1.17			0.85	1.24		
6	Fish	0.90	0.85	0.85	1.18	0.82	1.11	0.84	1.19			0.80	0.97	0.78	1.04
8	thyroid 96 mgm.	0.85	0.93	0.80	0.99	0.87	0.92*								
						0.78	1.23	0.73	1.19						
5	Av. dose	0.91	0.92			0.73	1.06	0.77	1.08						
Z		0.86	0.90	0.71	0.85	0.69	1.02	0.75	1.17	0.78	1.25	0.81	1.25		
K		0.93	1.01	0.77	1.16	0.74	1.38	0.78	1.17	0.75	1.20	0.84	1.10†		
3A		0.87	0.92	0.77	1.13	0.81	1.09	0.84	0.98	0.90	1.05	0.87	0.91		
5A		0.84	0.93	0.79	1.13	0.73	1.15	0.70	1.17	0.79	0.90	0.86	0.85		
Ave.		0.88	0.98	0.78	1.07	0.77	1.12	0.77	1.14	0.81	1.10	0.84	1.02		

* Re-injected with larger dose.
† Died on 8th day.

neutralized NaOH. These experiments are divided into four series, each of which is discussed separately below.

Neutralized NaOH solution. As a control on the handling and injection of the rats, neutralized NaOH solution was injected, in the same amount and manner as were the thyroid preparations. Six rats were so treated and in no instance was there any significant change in R.Q. up to 7 days after the injection. A slight fall in oxygen consumption during the experiment was probably attributable to adaptation.

Thyroxine. Synthetic thyroxine (Roche-Organon) was injected into 5 rats at two dose levels, 1.0 mgm. and 0.2 mgm. There appeared to be no appreciable difference between the effects of these two doses. Maximum effects on oxygen consumption and R.Q. were seen within two days after the injection.

Mammalian thyroid extracts. Commercial desiccated mammalian thyroid was extracted and injected in the manner described above. In two experiments 130 mgm. were given each time. There was a marked elevation of oxygen consumption, and a fall in R.Q. on the first day after the injection which continued until beyond the fourth day.

Fish thyroid extract. Extracts of parrot fish thyroid glands prepared in the manner already described were injected intra-abdominally into seven rats. The amount of dried gland extracted for this purpose varied between 69 and 110 mgm. In rat No. 8, 69 mgm. failed to produce an elevation in oxygen consumption or a fall in R.Q. by the second day after injection. Possibly this dosage was too small. On the second day after the original injection, therefore, the same animal was reinjected with 98 mgm. of dried extracted gland. Two days later it showed a marked rise in oxygen consumption and a fall in R.Q. In all other instances an elevation in oxygen consumption and a fall in R.Q. were observable within the first or second day after injection. In most cases oxygen consumption was still elevated on the seventh day, although the R.Q. usually had returned to the pre-injection level by that time.

Figure 1 shows graphically the average percentage changes in oxygen consumption and R.Q. All thyroid preparations produced increases in oxygen consumption and decreases in R.Q., while those rats injected with neutralized NaOH solution showed no percentage change in R.Q. and a perceptible decrease in oxygen consumption. This decrease is undoubtedly due to the adaptation of the animal to the respiratory chamber. As might be expected, the effect of synthetic thyroxine on oxygen consumption is more marked and of shorter duration than are the effects of extracts of fish and mammalian thyroid. Fish and mammalian thyroid injections increased oxygen consumption about 25 per cent at the maximum and the effects persisted for at least 10 days. There is a suggestion that fish thyroid has a somewhat more prolonged action than has mammalian thyroid. This also seems to be true of the effect on R.Q., which was still depressed 10 days after fish thyroid injection, but after mammalian thyroid injection it had returned to the pre-injection level within 7 days. The quantitative differences in the effects of synthetic thyroxine and extracted mammalian and fish thyroid are undoubtedly due to variations in the rate of absorption of the injected material.

No attempt has been made to determine the relative strengths of fish and mammalian thyroid. Since the amounts of the two extracts producing similar effects

were of the same order of magnitude, they are probably of the same approximate strength.

Figure 2 shows the effect of fish thyroid extract upon the weight of injected rats as compared to the effect of neutralized NaOH. In the four cases graphically por-

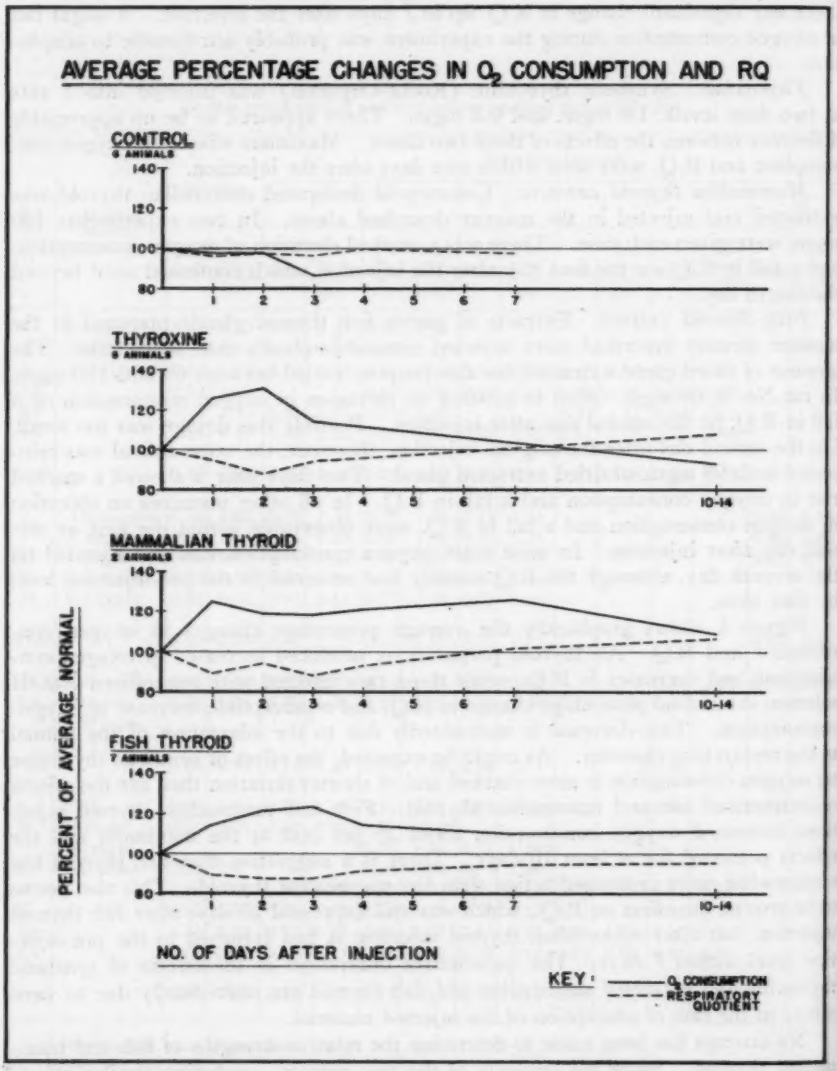

FIGURE 1. Showing the effect of thyroid extracts of fish and mammalian origin, as well as effect of thyroxine, on the oxygen consumption and respiratory quotient of white male rats.

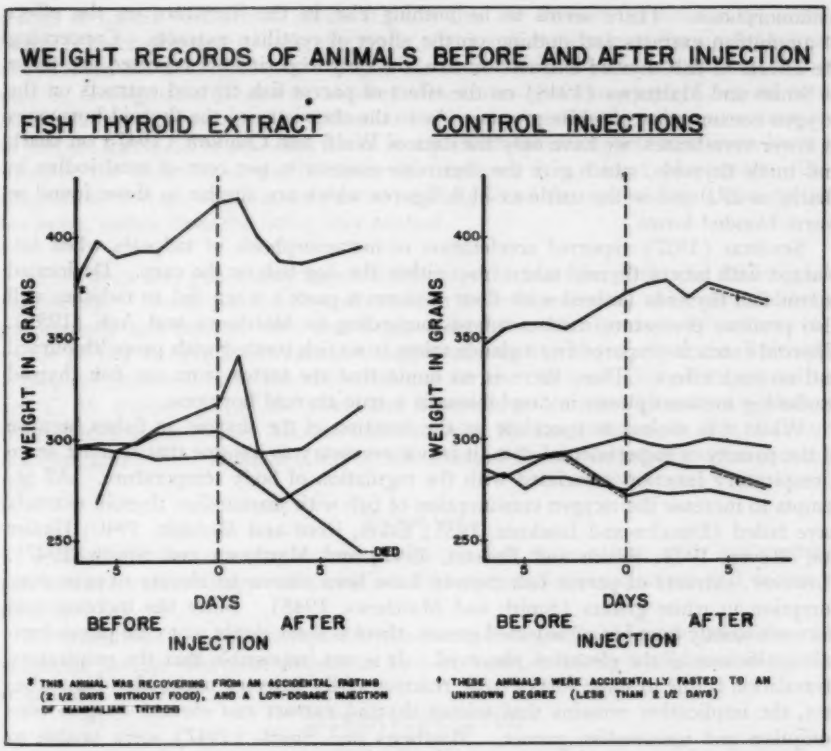

FIGURE 2. Showing effect of parrot fish thyroid extracts on the weight of white male rats.

trayed, fish thyroid extract invariably produced a marked fall in weight on the first or third day after injection, the fall persisting for at least 4 days. In most instances the weight was not recovered until at least one week after the injection. In those rats which were injected with NaOH solution, the weight of the animal following injection was, beyond the random variations, no different from the pre-injection value. While the effects of synthetic thyroxine and mammalian thyroid extract are not shown in this figure, their effects on weight are the same as that of the fish thyroid extract.

DISCUSSION

Data on the effect of thyroid extracts derived from the glands of lower vertebrates are virtually non-existent. The work done, however, is adequately considered in three excellent reviews on the comparative physiology of the thyroid which have appeared recently (Fleischmann, 1947; Goldsmith, 1949; and Lynn and Wachowski, 1951). These papers cover the subject so thoroughly that there is little point in reviewing the literature here except to summarize it briefly. Fleischmann (1947) notes only the effect of transplanted amphibian glands on amphibian

metamorphosis. There seems to be nothing else in the literature on the effect of amphibian extracts and nothing on the effect of reptilian extracts. Concerning the effects of fish thyroid extracts on fish, the only experiments reported are those of Smith and Matthews (1948) on the effect of parrot fish thyroid extracts on the oxygen consumption of white grunts. As to the chemistry of the thyroid hormones of lower vertebrates, we have only the data of Wolff and Chaikoff (1947) on shark and turtle thyroids, which give the thyroxine content in per cent of total iodine in sharks as 27.9 and in the turtle as 31.8, figures which are similar to those found in warm-blooded forms.

Sembrat (1927) reported acceleration of metamorphosis of tadpoles when implanted with bits of thyroid taken from either the dog-fish or the carp. Desiccated parrot fish thyroids (mixed with flour to form a paste) when fed to tadpoles will also produce premature metamorphosis according to Matthews and Ash (1951). Thyroid extracts prepared from glands taken from fish treated with propylthiouracil had no such effect. Thus, there is no doubt that the factor from the fish thyroid producing metamorphosis in amphibians is a true thyroid hormone.

While it is useless to speculate on the function of the thyroid in fishes because of the paucity of experimental data, it is not necessary to assume that it must serve a respiratory function associated with the regulation of body temperature. All attempts to increase the oxygen consumption of fish with mammalian thyroid extracts have failed (Drexler and Issekutz, 1935; Etkin, Root and Mofshin, 1940; Hasler and Meyer, 1942; Smith and Everett, 1943; and Matthews and Smith, 1947). However, extracts of parrot fish thyroid have been shown to elevate oxygen consumption in white grunts (Smith and Matthews, 1948). Since the increase was not consistently found in all injected grunts, there is some doubt as to the physiological significance of the elevation observed. It is not impossible that the respiratory stimulation is due to some sort of toxic reaction of the injected material. Nevertheless, the implication remains that teleost thyroid extract can elevate oxygen consumption and mammalian cannot. Matthews and Smith (1947) were unable to observe any change in oxygen consumption in Fundulus when injected with thiourea over a period of 5 to 6 days. It is possible, of course, that longer treatment with thiourea might have produced a different result. Further studies are necessary, including observations on the effect of fish thyroid extracts and thiourea on the R.Q. of fishes, before a final conclusion can be drawn.

There seems little doubt that the increase in respiratory metabolism observed in white rats after injection of thyroid extracts of the parrot fish thyroid is due to increased cellular oxidation. The exact parallelism between the effect of thyroid extract of mammalian origin as compared to that of fish origin leaves little room to question that the two are much alike in their physiological effects in the mammal. This view is strengthened by the fact that synthetic thyroxine behaves in the same way. The rise in oxygen consumption, fall in R.Q. and decrease in weight all follow the same pattern, regardless of the source of the thyroid hormone. This finding, coupled with the observations that (1) both desiccated fish thyroids and extracts of these glands will produce premature metamorphosis in amphibians, (2) the fish thyroid will concentrate iodine, and (3) chemically the thyroids of those lower vertebrates that have been studied (the shark and the turtle) are very similar to the

glands of higher vertebrates, makes it likely that the thyroid hormone of all vertebrates is much the same. It is, therefore, obvious that the teleost during the course of evolution has not sufficiently changed the nature of its thyroid hormone so that it may be distinguished from that of other vertebrates by ordinary physiological tests on amphibians and mammals. Whether the hormone still serves the same function in teleosts that it did in ancestral forms is at present unknown, just as it is not known whether the function of the hormone in mammals is a recent adaptation. The adaptation, if true, would appear to be on the part of the mammalian tissue to the hormone, rather than the other way around.

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Summary

1. The effect of injecting extracts of parrot fish thyroid into adult, male white rats was to increase oxygen consumption, decrease respiratory quotient and decrease weight.

2. Injections of synthetic thyroxine or extracts of desiccated mammalian thyroid produced similar effects in respect to time of onset, intensity and duration of the responses.

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